Monoamine influences in cerebellar memory consolidation

Michael Longley

Department of Neuroscience, Physiology and Pharmacology

University College London (UCL)

2016

Thesis submitted to University College London for degree of Doctor of Philosophy

I, Michael Longley confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

An association between climbing fibre and mossy fibre/parallel fibre inputs to the Purkinje cell is critical for cerebellar learning. In addition to these two major afferent systems, the cerebellum also receives a range of neuromodulatory inputs; most prominent are the noradrenergic and serotonergic afferents. Early theoretical and empirical accounts support a role for noradrenergic input as providing an essential third, consolidation signal in learning. In comparison to the glutamatergic afferents very little is known about the anatomy, physiology and behavioural aspects of the neuromodulatory afferents.

The distributions by cell type of β_1- and β_2-adrenoceptors in the cerebellar cortex and nuclei and of α_1-adrenoceptors in the cerebellar cortex, are shown for the first time. Earlier work demonstrated the necessity for β-adrenoceptor activation in consolidation of classical conditioning of the nictitating membrane response (NMR). Here, a dissociation of β_1- and β_2-adrenoceptor expression was shown. β_1-adrenoceptors are restricted to Purkinje cells and β_2-adrenoceptors are restricted to Bergmann glial cells.

The cerebellar cortical distributions of noradrenergic and serotonergic afferents were compared. In cortical vermis, individual noradrenergic afferents were limited in their medial-lateral extent to less than 300 μm but were more extended in the rostral-caudal plane by up to 800 μm. Serotonergic afferents ran orthogonal to the noradrenergic afferents, with extents up to 900 μm in the medial-lateral plane but less than 200 μm in the rostral-caudal plane.

Recent work has demonstrated a critical role for Purkinje cell $mGlu_7$ activation in regulating the pause in Purkinje cell simple spike activity believed to be the cellular mechanism underpinning the conditioned eyelid blink/ NMR.

Attempts were made to assess the specific function of the β_1-adrenoceptor and $mGlu_7$ in consolidation and performance of NMR conditioning, respectively. However, methodological constraints left these questions unresolved.

It is concluded that the noradrenaline consolidation signal may target limited cortical territories and modulate Purkinje cells or Bergmann glial cells. In contrast, the serotonin signal is diffuse and targets multiple cortical regions simultaneously to fulfil a role in cerebellar processing distinct from that of noradrenergic signalling

Acknowledgements

First and foremost I would like to thank my supervisor Professor Christopher Yeo for his guidance and encouragement throughout my time in his lab, he has set a very good example of how to be a good scientist and academic, an example I hope I will be able to follow in my own career. I especially want to thank him for the time he has given up to provide me with invaluable feedback in producing this thesis, I hope that after a period of consolidation I will be able to implement the advice he has given me on good scientific writing and proper grammar in my own future writing.

I would also like to thank all the members of the lab I have work with over the years, who have all enriched my experience; including Maria Andres-Alonso (Maru) and Hadleigh Cuthbert who were both very helpful working with me to develop ideas and protocols, as well as being very good company. Also, Jamie Ballard, my fellow MSc student who kept me laughing throughout that year, including the last, very stressful month.

Thanks also to Professor Patrick Anderson who taught me so much of what I know about immunohistochemistry and was so generous with his time and lab space. Also, Professor Steve Edgley and Jack Curtis in Cambridge for hosting me and providing the space to do the behavioural work presented here.

Thank you to Tatum Ward who was there to provide support throughout the vast majority of my PhD and was a so wonderful at helping me forget about work when I needed to. Thank you to my twin brother David, who let me talk at him for hours at a time whenever I was mulling over a problem.

Finally, I would like to thank all of my friends and family for their love and support.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AIP Anterior Interpositus nucleus

BC Basket cell

BGC Bergmann glial cell

CF Climbing fibre

CS Conditional stimulus

CR Conditional response

DBH Dopamine β-hydroxylase

EBR Eyelid blink response

GC Granule cell

GCL Granule cell layer

GoC Golgi cell

ICP Inferior cerebellar peduncle

IHC Immunohistochemistry

IR Immunoreactivity

ISH In-situ hybridization

LRN Lateral Reticular nucleus

LGC Lugaro cell

MCP Middle cerebellar peduncle

MF Mossy fibre

ML Molecular layer

MLI Molecular layer interneuron

NMR Nictitating membrane response

NRTP Nuclear Reticularis Tegmenti Pontis

OKR Optokinetic reflex

PC Purkinje cell

PCL Purkinje cell layer

PF Parallel fibres

PN Pontine nuclei

RN Red nucleus

SC Stellate cell

SCP Superior cerebellar peduncle

UBC Unipolar brush cell

UR Unconditional response

US Unconditional stimulus

VOR Vestibulo-ocular reflex

Chapter 1: Cerebellar structure and function: implications for cerebellar learning

1.1 Functional anatomy of the cerebellum

The cerebellum is highly conserved through evolution, with homologous structure through all vertebrate species. Although its structural details vary among different vertebrate taxa, many aspects of its internal cytoarchitecture and input and output relationships are common. These include, in particular, the topographical organisation of sensory inputs through climbing fibres that innervate proximal portions of Purkinje cells and a molecular layer where Purkinje cells extend their apical dendrites to receive input across a range of modalities from the parallel fibres of granule cells (Bell, 2002). This high level of structural conservation suggests a common role for the cerebellum with survival importance for all vertebrates.

Through the work of experimental physiologists such as Rolando, Fluorens, Luciani and Dalton (see Glickstein and Doron, 2008; Fine et al, 2002) and the observations of clinicians including Babinski and Holmes (see Dow and Moruzzi, 1958; Fine et al, 2002) the crucial role for the cerebellum in the coordination of voluntary movements was established. A separate line of investigation established the importance of the cerebellum in the regulation of reflex motor behaviours (see Bloedel and Bracha, 1992; Welsh and Harvey, 1992). Later recent experimental work implicated the cerebellum in the learning of voluntary movements (see Thach, 1998a and b) and the adaptation of reflexes (Ito et al, 1974; Robinson et al, 1976; Yeo et al, 1985a, b; Yeo and Hesslow, 1998). Most recently, claims have been growing that the cerebellum may play a role in cognitive processes but the extent and nature of this involvement is still uncertain (Thach, 1998a, b, Schmamann and Sherman, 1998; Koziol et al, 2014).

1.1.1 Cerebellar gross anatomy

The cerebellum can be considered as having three main components. A three-layer cortex forms the surface of the cerebellum. Below the cortex is the cerebellar white matter which comprises afferent and efferent fibres of the cerebellar cortex and of the cerebellar nuclei. The cerebellar nuclei are the third component. They are embedded within the white matter and they are major relays for information from the cerebellar

cortex. All regions of the cortex project to the cerebellar nuclei, except for some vestibular-related cortical regions that project instead to the vestibular nuclei. The cerebellar influence upon multiple brain regions is entirely by projections from the cerebellar and vestibular nuclei.

The cerebellar cortex in mammals has multiple folia separated by fissures. Two very prominent fissures divide the cortex into three anatomically defined lobes. The primary fissure divides the anterior from the posterior lobe and the posterolateral fissure divides the posterior from the flocculonodular lobe. These lobes were further subdivided by Larsell into 10 lobules; each lobule is made up of one or a small number of folia depending on the species (Larsell, 1952; 1953). The anterior lobe contains Lobules I-V, the posterior lobe contains lobules VI-IX and the flocculonodular lobe is Lobule X (see fig 1.1). At the midline, the long axis of each folium and of each lobule is orthogonal to the sagittal plane and with increasing laterality this axis deviates from the orthogonal, which makes the tracing of foliar continuity complex.

In addition to this rostrocaudal organization, by lobe, lobule and folium, the cerebellar cortex can also be divided into three regions in the mediolateral plane. From medial to lateral these regions are the vermis, paravermis and hemispheres. The hemispheres include the flocculus and paraflocculus. Larsell suggested that hemispheral lobules are continuities of the vermis lobules and thus, in Larsell's nomenclature, hemispheral lobules are denoted by the prefix H, for example the hemispheral extension of vermal Lobule VI is Lobule HVI. However other workers have designated unique nomenclature for hemispheral lobules (See fig 1.1. Bolk, 1906; Voogd and Glickstein, 1998; Glickstein et al, 2011). The cerebellar cortex can be further divided mediolaterally into longitudinal zones. These zones were first identified by Voogd (1969) on the basis of histological staining of white matter fascicles. Subsequent increasingly detailed studies have shown that these zones are signs of a subsystem of mediolaterally organised functional zones (see Apps and Hawkes, 2009; Voogd, 2011 for reviews) which themselves can be further divided into microzones on the basis of their nuclear and olivary connections (Oscarsson, 1979. For a more detailed discussion of mediolateral zonation see section 1.1.5).

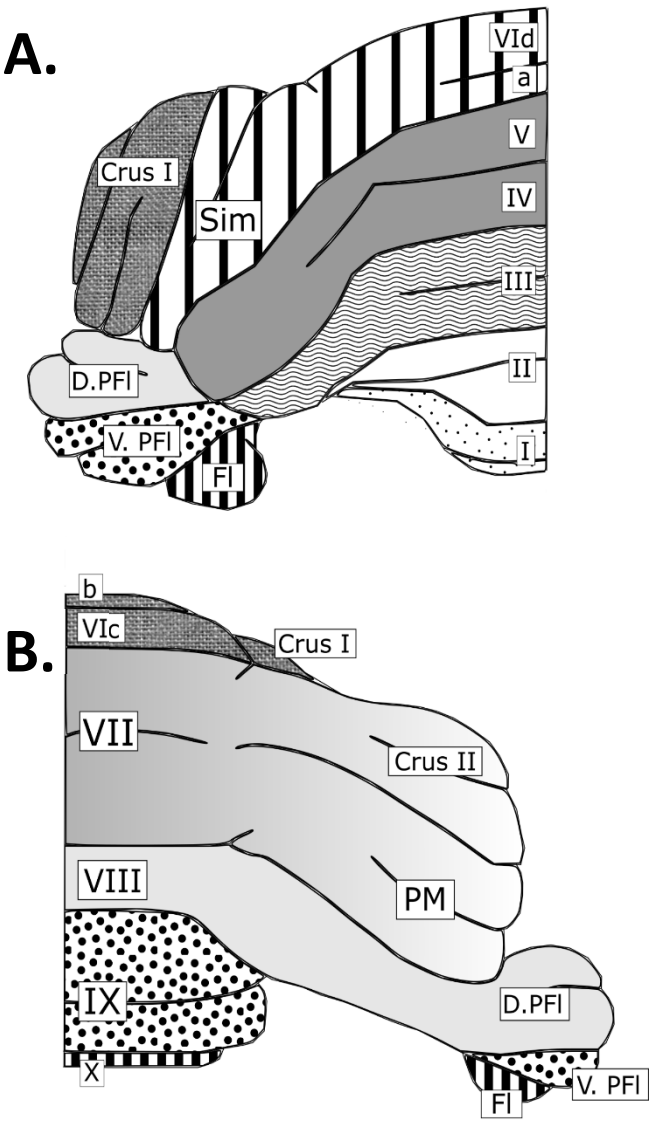


Fig. 1.1: Anatomy of lobules of the cerebellum in rats. Vermis and hemisphere lobules filled with the same pattern indicate continuities of vermis lobules into the hemispheres. **A) Posterior view.** Lobules IV/V and III continuities with the hemispheres. Vermis lobule VId/a continue into Lobulus simplex (Sim). **B) Anterior view.** Vermis lobule VIb/c continues into Crus I. Lobule VII continues into Crus II and paramedian lobule (PM). Lobule VIII continues to dorsal paraflocculus (D.PFl). Lobule IX continues to ventral paraflocculus (V.PFl). Lobule X continues to Flocculus (Fl). Adapted from Larsell (1952).

The rostrocaudal lobular organisation is easily identified anatomically but functionally it is of secondary importance to the mediolateral zonal organisation, which can be identified by olivary and nuclear connectivity. Even though the zonal and microzonal organization of the cerebellum is largely unseen at the gross anatomical level, it is the major organisational principle and a critical determinant of cerebellar function (See section 1.1.5 for a comprehensive discussion of mediolateral zones).

1.1.2 Functional anatomy of the cerebellar cortex: A uniform cellular organisation that implies a uniform organisation of information processing

The cerebellar cortex consists of three distinct layers; the granule cell layer internally, the outermost molecular layer and the Purkinje cell layer which lies between these two. The cerebellar cortex includes a relatively small number of distinct cell types, many of which maintain a stereotyped distribution across the entire cortical surface and whose axonal and dendritic processes maintain a specific arrangement in relation to other cell types and the foliated architecture of the cortex (see Fig. 1.2 for simplified diagram of the cell types of the cerebellar cortex and their connections). This stereotyped distribution of soma and processes of the cerebellar cortex makes its cytoarchitecture nearly identical from region to region. The uniform circuitry of the cerebellar cortex is a foundational principle of most prominent theories of cerebellar function (see Chapter 2, Marr, 1969; Albus, 1971; Ito, 1972; Gilbert, 1974).

The primary and only output neurons of the cortex are the inhibitory Purkinje cells (PC) whose soma lie in a monolayer, the Purkinje cell layer (PCL), between the molecular and granule cell layers. Purkinje cell dendrites are highly ramified in one plane to create a large fan-like dendritic arbor that is always aligned perpendicular to the long axis of the folium. Purkinje cell axons project to the underlying cerebellar nuclei and receive input from the two major afferent inputs to the cortex, the climbing fibres (CF) directly and the mossy fibres (MF) indirectly via the ascending axons and parallel fibres from the underlying granule cells.

Mossy fibres contact the excitatory granule cell (GC) via large synaptic specializations called mossy fibre rosettes. Each rosette contacts the dendrites of multiple granule cells and each granule cell receives input from a small number of MFs that is thought to be four in most instances (Eccles et al; 1967; Jakab and Hamori 1988). Granule cells have extremely small soma (most with a diameter of 5-6 μm in the rat, Palay and Chan-Palay, 1974) and they are packed very densely (17700 per mm^2 compared to 936 per mm^2 for PCs. Harvey and Napper, 1988) so that a very large number populate the granule cell layer (GCL). They are the most numerous cell type in the brain by a very large majority (an estimated 50 billion or ¾ of the total neuronal population in humans. Llinas et al, 2004). Each GC gives off an ascending axon with synaptic contacts upon the immediately overlying Purkinje cells. The ascending axon synapses constitute only

a small proportion of the total GC-PC synapses (~5% Huang et al, 2006; Lu et al, 2009) and their transmission and plasticity properties differ from those of the parallel fibres (Sims and Hartell, 2005; Sims and Hartell, 2006). The ascending axon enters the molecular layer (ML) and rises through it to a height related to the depth of its source granule cell in the GCL. Ascending axons from the deep parts of the GCL rise only to the deep ML, but those from the more superficial parts of the GCL rise higher in the ML. They then bifurcate to form a T-shaped branch that gives rise to two parallel fibres (PF) extending in opposite directions for several millimetres along the long axis of the folium (estimates of 4-5 mm in adult rat, Napper and Harvey, 1988; Pichitpornchai et al, 1994). By imaging PC activation dependent changes in fluorescence, Coutinho et al (2004) showed in mice that electrical stimulation of PFs directly or by stimulation of mossy fibres caused a longitudinal beam-like propagation of PC activation for 1.5 mm in each direction. Because the planar PC dendritic arbors are arranged perpendicular to the folial long axis, PFs pass through the dendritic trees of many hundreds of PCs, forming synapses on the dendritic spines of the intermediate and distal dendrites of many, but not all, Purkinje cells along their length (~50%, Harvey and Napper, 1988). Due to the large ratio of granule cells to PCs (an estimated 274:1 in the rat cerebellum, Harvey and Napper, 1988) and the extensive reach of PF synaptic contacts along the folia there is a very large convergence of GC to PC input with each PC receiving a prodigious number of glutamatergic PF inputs (approximately 150,000 in the rat. Harvey and Napper, 1991).

The standard mode of activity for PCs is simple spike firing, which occurs at highly variable rates from 40-100 Hz or more and is determined by a combination of spike-generating mechanisms intrinsic to the PC (Hausser and Clarke, 1997) and granule cell input both directly through PF-PC excitatory (and ascending axon-PC) inputs and indirectly through PF synaptic drive on inhibitory interneurons (see below) that are presynaptic to PCs (De Zeeuw et al, 2011). The second mode of activation of PCs is the CF-evoked complex spike that is much less common then the simple spike and occurs with an average frequency around 1 Hz. It is a strong and long-lasting depolarization of the PC characterised by a single action potential followed by several smaller secondary spikes (Thach, 1967). Individual CF branches contact a single PC and adult PCs receive input from only a single CF but each CF-PC connection consists of 1000s of active synaptic contacts across its dendritic tree such that a single CF action can depolarise the entire PC (Eccles et al, 1967; Palay and Chan-Palay, 1974). The coincidence of the distinct post-synaptic effects of PF and CF activity at the PC are

proposed to form the underlying generator of learning-related plasticity in the cerebellar cortex (Marr, 1969; Albus, 1971; Ito, 1972; Gilbert, 1974).

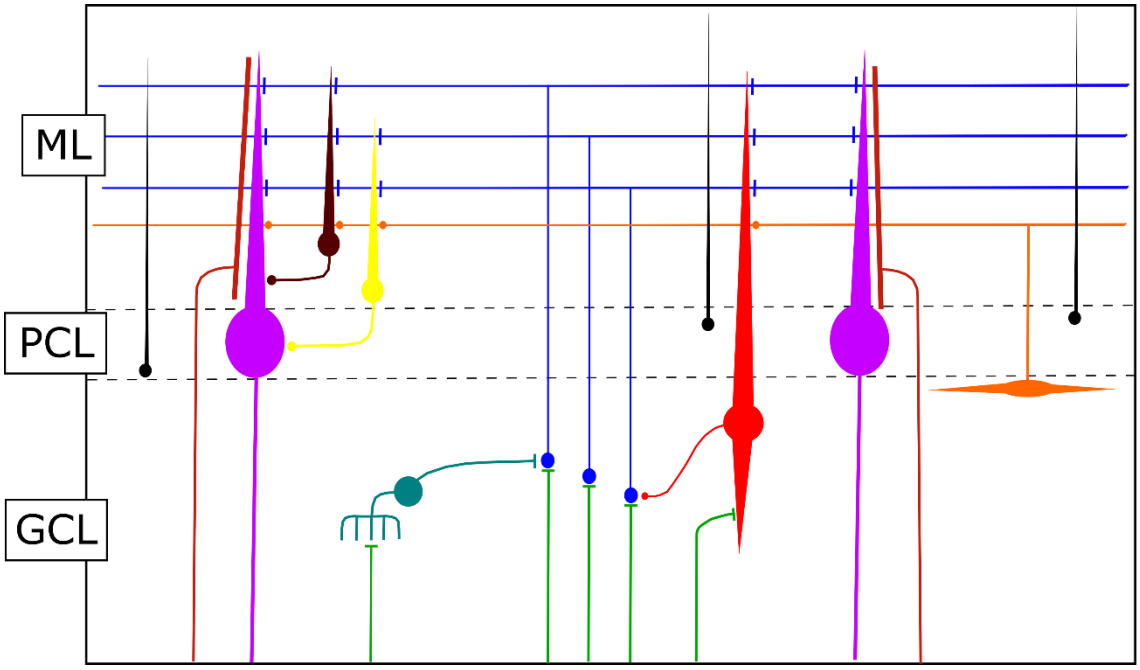


Fig. 1.2: Simplified diagram showing cell types of the cerebellar cortex and their connections. Excitatory synapse represented by straight line. Inhibitory synapse represented by circle. Mossy fibre ( ); Climbing fibre ( ); Purkinje cell ( ); Basket cell ( ); Stellate cell ( ); Golgi cell ( ); Lugaro cell ( ); Unipolar brush cell ( ); Bergmann glia ( ). GCL: Granule cell layer, ML: Molecular layer, PCL: Purkinje cell layer.

In addition to GCs the granule cell layer also contains Golgi cell (GoC) inhibitory interneurons. Golgi cell axons are highly ramified within the GCL and provide inhibitory inputs to the dendritic region of thousands of GCs. These GoC inputs form a tripartite processing structure with the MF rosette and GC dendrites known as the glomerulus. Golgi cells have basolateral dendrites restricted to the GCL and an ascending dendritic tree which reaches into the ML (Eccles et al, 1967). The basolateral dendrites receive excitatory input from MFs (Kanichay and Silver, 2008) and the ascending dendrites receive input from PFs (Palay and Chan-Palay, 1974). It is traditionally believed that the Golgi cell draws on PF signalling to its ascending dendrite to provide a feedback gating mechanism on MF-GC synaptic transmission (Eccles et al, 1966; Eccles et al, 1967). Additionally MF input to its basolateral dendrite provides a mechanism for feedforward or lateral inhibition of the same synapse (Kanichay and Silver, 2008;

D'Angelo and De Zeeuw, 2009). Through these mechanisms GoCs are ideally placed to modulate information transmission from the MFs to Purkinje cells.

In addition to the Purkinje cell dendritic trees the molecular layer also contains two types of molecular layer interneuron (MLI) – the basket and stellate cells (BC and SC). Both cell types receive excitatory input from PFs and they provide inhibitory input to PCs. Basket cells are localized in the deeper levels of the ML and mainly target their inhibitory synaptic input to the PC soma and axon initial segment. In contrast, the stellate cells are found through the middle and superficial levels of the ML and largely target their inhibitory synaptic input to the PC dendrites. Additionally MLIs provide inhibitory input to other nearby MLIs (Eccles et al, 1967; Palay and Chan-Palay, 1974; Jorntell et al, 2011) and they are also believed to provide inhibitory input to the ascending dendritic section of GoCs (Dumoulin et al, 2001).

These five cell types: Purkinje cells, granule cells, Golgi cells, basket and stellate cells garner the most interest in classical accounts of cerebellar physiology (Eccles et al, 1967) and feature most prominently in theoretical accounts of cerebellar function (e.g. Marr, 1969; Albus, 1971). However, in addition to these five neural types, there are a number of other cell types present in the cerebellar cortex that have attracted less interest. These include the inhibitory Lugaro cell (LG), several other neuron types such as the globose or chandelier cells that are morphologically distinct from Lugaro cells but probably functionally comparable (Laine and Axelrad, 2002) and the excitatory unipolar brush cell (UBC). The cerebellum has a normal complement of glial cell types but also a unique, cerebellum-specific type – the Bergmann glial cell (BGC), a radial glial cell that is still present in the adult cerebellum and functions similar to astrocytes elsewhere in the adult (De Zeeuw and Hoogland, 2015).

The Lugaro cell is a mixed, GABAergic/ glycinergic inhibitory neuron (Dumoulin et al, 2001) found in the upper granule cell layer directly below the PCL. It is a fusiform cell with dendrites oriented transverse to the long axis of the folium and travelling for long distances in both directions just below the PCL. The Lugaro cell has two sets of highly ramified axons that target the ML and, potentially, the PCL: the first runs transverse to the long axis of the folium similar to its dendritic orientation whilst the second travels along the long axis of the folium perpendicular to its dendritic arbor. The LGC is thought to be activated solely by 5-HT (Dieudonné and Dumoulin, 2000; Dean et al,

2003). Purkinje cells (Dean et al, 2003), molecular layer interneurons (Laine and Axelrad, 1998) and Golgi cell ascending dendrites (Dieudonné and Dumoulin, 2000) in the molecular layer are postsynaptic targets of the LGC, consistent with the suggestion that 5-HT can have significant influence on information processing in the cerebellar circuit via these neurons.

Unipolar brush cells are the only non-GC excitatory neuron of the cerebellar cortex and are largely restricted to the vestibulocerebellum. They receive input from MFs at a distinctive brush-like dendrite and their axons branch before forming large, rosette-like terminations similar to those of MFs. As with MF rosettes, these UBC terminations also target multiple granule cell dendrites, forming structures similar to the glomeruli formed between MFs and granule cells and suggesting that UBCs may act as intermediaries that amplify the excitatory drive from MFs to granule cells (Kalinichenko and Okhotin, 2005). It has been reported that UBC outputs also target GoCs and other unipolar brush cells (Diño et al, 1999; 2000).

Bergmann glia are a specialised astrocytic glial cell type found exclusively in the cerebellar cortex. They have soma within the PCL and intermingled with the PC somas and processes that extend radially from the PCL through the ML to the pial surface where they terminate as end feet (de Blas, 1984; Reichenback et al, 1995; Castejon et al, 2002). Along the length of the BGC processes are specialised ramifications known as lateral appendages that ensheathe parallel and climbing fibre synapses onto PCs (Grosche et al, 1999; 2002) and closely appose CF and MLI synapses (Castejon et al, 2002). The traditional view of glial cells is as passive support cells providing trophic support for neurons. However, BGCs express a large repertoire of neurotransmitter receptors (Bergles et al, 1997; Verkhratsky et al, 1998; De Zeeuw and Hoogland, 2015) including AMPA receptors on their lateral appendages (Matsui and Jahr, 2004; Matsui et al, 2005), this suggests that BGCs may participate in information processing cooperatively with neurons in the cerebellar cortex. For example, stimulation of PFs in slice preparations leads to a major calcium influx into BGCs, which has a modulatory role in PC simple spike firing (Wang et al, 2012). Such evidence suggests that neuronal-BGCs interactions may make a significant contribution to signal processing in the cerebellar cortical microcircuit (further discussion in sections 2.4.2 and 5.2).

1.1.3 The cerebellar nuclei: output pathways and the O.C.N loop

Purkinje cells, the sole output neurons of the cerebellar cortex project almost exclusively to the cerebellar nuclei. The only exception is some Purkinje cells of the floccular-nodular cortex that project directly to the vestibular nuclei. From medial to lateral, the human cerebellar nuclei are the fastigial, emboliform, globose and dentate nuclei. In non-human species the emboliform and globose are recognised as the anterior and posterior interposed nuclei, respectively (AIN and PIN respectively), though their division if often indistinct anatomically so a single interposed nucleus is sometimes labelled. The majority of efferents to the fastigial nuclei originate from the vermis and their main output targets are the vestibular nuclei, nuclei of the reticular formation, thalamus and superior colliculus. The interposed nuclei receive most of their input from intermediate cortex (paravermis) and provide significant output to the red nucleus (RN), superior colliculus, nuclei of the reticular formation and the primary motor cortex (AIN) and premotor cortex, primary and supplementary eye-fields and parietal cortex via the thalamus. Finally the dentate nucleus receives the majority of its input from the lateral cortex (hemispheres) and most of its output is targeted to the primary and premotor cortex, primary and supplementary eye-fields and parietal cortex via the thalamus but also to the RN, superior colliculus and spinal cord (Fig. 1.3 for diagram of nuclei of the rat cerebellum. See Teune et al, 2000; Voogd et al, 2013 for review of efferent targets).

There are reported to be six distinct neuronal types within the cerebellar nuclei that can be distinguished by molecular, morphological and electrophysiological criteria (Chan-Palay, 1977; Uusisaari and De Schutter, 2011). These types include GABAergic/ glycinergic and non-GABAergic (putatively glutamatergic) interneurons and two glycinergic projection neurons with heterogenous distributions; one set found in the lateral cerebellar nuclei that project to the GCL (probably targeting GoCs) and another set within the fastigial nuclei projecting to the vestibular nuclei. The two remaining and best understood neuronal sub-types are the large glutamatergic projection neurons and a smaller GABAergic projection neuron (Nelson and Mugnaini, 1989, Fredette and Mugnaini, 1991; Uusisaari and De Schutter, 2011).

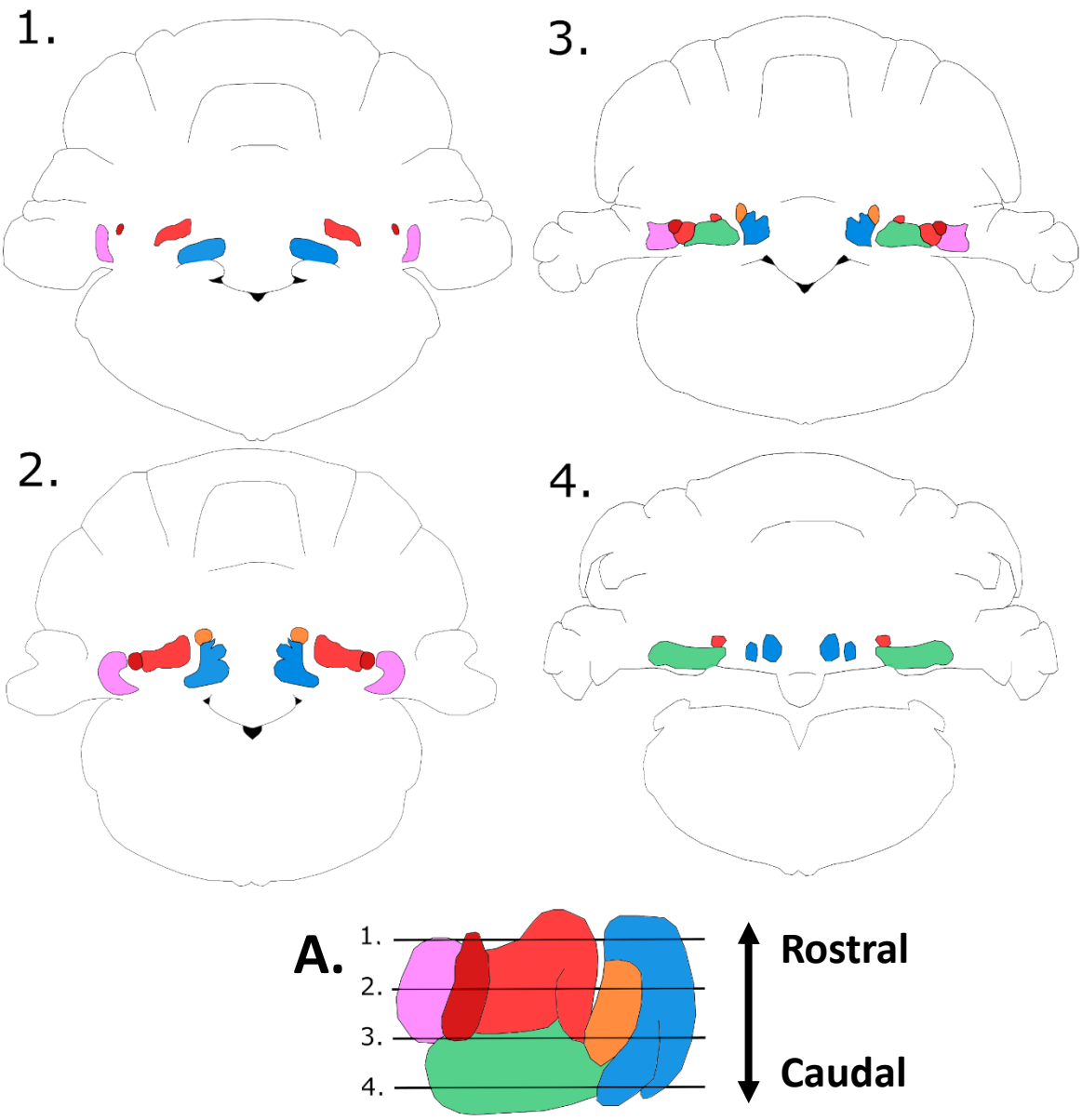


Fig. 1.3: Cerebellar nuclei: structure and position at different rostral-caudal positions in the rat cerebellum. A) Dorsal view of the cerebellar nuclei. Dentate nuclei (), Dorsolateral hump (), Anterior nuclei (), Posterior nuclei (), Fastigial nuclei (), Dorsolateral proterberance (). A) Levels of coronal sections 1-4. Adapted from Voogd et al (2013).

The glutamatergic projection neuron type receives significant axosomatic and axodendritic inhibitory drive from Purkinje cell axons (Chan-Palay, 1973). It is traditionally believed that the function of these projection neurons is to relay the results of information processing completed in the cerebellar cortex to their premotor targets in order that the cerebellum can exert control over movement. In keeping with this view, the main output targets of these neurons are motor and premotor structures with direct projections to spinal cord motor neurons, the superior colliculus, red nucleus and pre-motor thalamic nuclei (Toyama et al, 1970; Ito, 1984; Shinoda et al, 1985a, b; Tuene et al, 2000; Voogd et al, 2013).

The GABAergic cerebellar nucleus neuron subtype provides an inhibitory input to inferior olive neurons that give rise to the cerebellar cortical-projecting climbing fibres (Hesslow, 1986; Andersson and Hesslow, 1987a, b; Andersson et al, 1988). The detailed function of this pathway is still debated (Bengtsson and Hesslow, 2006) but it has been observed that those PC axons that target the large glutamatergic projection neurons also collateralize to target these GABAergic neurons (Teune et al, 1998). This pattern of connection suggests that both of these excitatory and inhibitory nuclear neuron types relay similar results of information processing in the cerebellar cortex for use by the IO and other cerebellar targets. This aspect of nuclear function will be discussed further.

Purkinje cell input to the cerebellar nuclei constitutes the majority of synaptic inputs but there are also two excitatory afferent inputs. These are from axon collaterals of the two major cerebellar afferents. Mossy fibre collaterals to the cerebellar nuclei originate from the pontine nuclei (PN), nucleus reticularis tegmenti pontis (NRTP) and lateral reticular nuclei (LRN) (Palay and Chan-Palay, 1974; Gerrits and Voogd, 1987; Shinoda et al, 1992; Mihailoff, 1993; Wu et al, 1999) and all MFs in the cerebellar nuclei are collaterals of fibres destined for the cerebellar cortex. No nuclei specific MFs have been observed. The different precerebellar nuclei contribute different numbers of nuclear collaterals. For example, almost all MFs traced from the NRTP and LRN give rise to nucler collaterals whilst only 1/5th of those traced from the PN do so (Shinoda et al, 1992; Shinoda et al, 1997; Wu et al, 1999). The second source of excitatory input to the nuclei are the climbing fibre collaterals (Palay and Chan-Palay, 1974; van der Want et al, 1989; Sugihara et al, 1996; Sugihara et al, 1999). More than 90% of CFs provide collateral input to the nuclei (Sugihara et al, 1999).

1.1.4 Mossy fibres: A diverse and divergent afferent system

Numerically, the most significant afferent inputs to the cerebellum are the glutamatergic mossy fibres, ultimately destined for the granule cell layer. Individual mossy fibres divide several times in the cerebellar white matter and each collateral can target widely disparate regions of the cerebellar cortex across the medio-lateral and rostro-caudal planes (Shinoda et al, 1992; King et al, 1998, Wu et al, 1999; Herrero et al, 2002). Once they reach the granule cell layer MFs collateralize further and terminate as ~20-30 rosettes (Palay and Chan-Palay, 1974; Jakab and Hamori, 1988) and each rosette provides synaptic input to a large number of GCs (estimates of between 15-50. Fox et al, 1967; Eccles et al, 1967; Jakab and Hamori, 1988). Eccles et al (1967) estimated a divergence ratio of one mossy fibre to between 400-600 granule cells and each granule cell receives axodendritic input from approximately four mossy fibres (Palay and Chan-Palay, 1974, Jakab and Hamori, 1988; Jorntell and Ekerot, 2006). Thus, the MF-GC pathway is characterised by a small amount of convergence of MFs targeting GCs and a very large amount of divergence in the MF afferent pathway. Similar to its cortical projection the MFs provide multiple collaterals that terminate widely in the cerebellar nuclei receiving term from a single MF collateral (Wu et al, 1999)

Mossy fibres enter the cerebellum via the inferior, middle and superior cerebellar peduncles (ICP, MCP and SCP) with a significant majority through the MCP. They provide GCs with information encompassing a range of modalities including motor and sensory information from cortical regions and sub-cortical nuclei. The afferents originate from a number of precerebellar nuclei including the LRN and NRTP as well as some spinal cord regions (Ito, 1984). However, the largest number of MFs is from the pontine nuclei which act as relay for cerebro-cerebellar signalling and all of these pontocerebellar MFs enter the cerebellum through the MCP.

1.1.5 Climbing fibres and the functional compartmentalisation of the cerebellum

The climbing fibre afferent system is the second largest afferent input to the cerebellum and although it has many fewer fibres than the MF afferent system it is an equally critical component of cerebellar function. Climbing fibres originate solely from the inferior olive nucleus (IO) and enter the cerebellum through the ICP. The chief target of CFs is the cerebellar cortex but they also collateralize within the cerebellar white matter and innervate the cerebellar nuclei in a manner similar to that for MFs.

Climbing fibres terminate in the ML and exert a direct influence over PC activity through synapses on the Purkinje cell dendritic tree. Each olivary axon gives rise to an average of seven climbing fibres (range: 2-17; Sugihara et al, 2000). The CFs originating from a single olivary neuron can terminate within a single lobule or in multiple contiguous lobules but can also be distributed widely, terminating in multiple non-contiguous lobules (Sugihara et al, 2001). Despite their broad rostral-caudal distribution Sugihara et al (2001) observed that CFs originating from a single olivary neuron terminate within narrow medial-lateral strips and the termination patterns of CFs from neighbouring olivary neurons are also restricted within a narrow medial-lateral strip; this pattern is likely to underpin the longitudinal zonal structure of the olivo-cerebellar projection (see the remainder of section 1.1.5 for discussion of longitudinal zones). Each individual CF targets only a single PC but the extensive synaptic contacts formed between each CF and the target PC allows individual olivary neurons are able to exert a significant effect, in the form of complex spikes, on the firing of a handful of Purkinje cells. Additionally, CFs also modulate MLI activity, potentially through spillover of glutamate from CF to Purkinje cell synapses (Ekerot and Jorntell, 2001; Jorntell and Ekerot, 2003).

Regional differences in cytoarchitecture have provided a strong basis for division of the cerebral cortex and these differences are loosely related to cerebral cortical functional organisation (originally by Korbinian Brodmann, see Zilles and Amunts, 2010) but its uniform cytoarchitecture has ruled out this approach for the cerebellum. Instead the functional specialisations of different regions of the cerebellum can be determined by the distributions of functionally distinct afferents and by the efferent targets of each region.

The climbing fibre terminations in the cerebellar cortex are organised in anatomically delimited, functionally relevant zones. Climbing fibre terminations are organized in strips that run transverse to the long axis of the folium and so they are oriented parasagittally close to the midline vermis (Groenewegen and Voogd, 1977; Groenewegen et al, 1979; Oscarsson, 1979). Climbing fibre terminal strips are relatively narrow in their mediolateral extent (~1-2 mm) but are extensive in their rostrocaudal extent with several strips spanning the entire cerebellar cortex. These strips will henceforth be referred to as longitudinal zones or zones. Each longitudinal zone receives input from a distinct region of the IO. The parasagittal organisation of the olivo-cerebellar projection is maintained in the cortico-nuclear projection such that

Purkinje cells in specific zones project to discrete regions within their target cerebellar nuclei (reviewed in Voogd and Bigaré, 1980; Buisseret-Delmas and Angaut, 1993).

This zonal pattern was originally observed by Voogd and colleagues (Voogd et al, 1969; Groenewegen and Voogd, 1977; Groenewegen et al, 1979). The white matter was compartmentalised based on the staining of fascicles containing efferent axons from overlying PCs and ascending afferents. The borders of these zonal compartment markers in the white matter were projected upwards to divide the cerebellar cortex into zones. This zonation was also seen to be maintained in the terminations of the PC axons upon the cerebellar nuclei (Voogd and Bigaré, 1980). These original observations were extended in experiments where CF trajectories were traced by examining degeneration patterns induced by lesions of specific olivary sub-nuclei to reveal that olivary axons also passed through the relevant white matter compartments as defined by PC-nuclei projections (Voogd, 1969). Based on these original anatomical observations (Voogd, 1969; Groenewegen and Voogd; 1977; Groenewegen et al, 1979) Voogd partitioned the cerebellar cortex into seven distinct zones on each side of the midline. From medial to lateral, these were labelled: A, B, C_1, C_2, C_3, D_1 and D_2.

Contemporary with the anatomical studies of Voogd and his colleagues, electrophysiologists began to see a similar pattern of sagittal organisation of the olivo-cerebellar projection using functional/stimulation studies of the climbing fibre projections to PCs and of the PC projections to the cerebellar nuclei (Oscarsson, 1979; Trott and Armstrong, 1978a, b).

More precise anatomical tracing methods have subsequently confirmed the compartmentalised olivary-cortical-nuclear connectivity (Dietrichs and Walberg, 1979; Voogd and Bigaré, 1980; Trott and Armstrong, 1987a; Buisseret-Delmas and Anguat, 1993; Sugihara et al, 2001; Sugihara and Shinoda, 2004). The combination of these methodologies reveals a set of zones which are nearly completely congruent as they are revealed by anatomical and electrophysiological methods. A current schema recognizes, from medial to lateral: zones A, AX, X, B, A2, C1, CX, C2, C3, D1, DO and D2 in the rat (Fig. 1.4. See Apps and Hawkes, 2009). Although studies in other mammalian species have demonstrated that some specific details can vary (for example, a Y zone can be identified in the cat, Ekerot and Larson, 1982) the general organisational pattern is well conserved across species (Apps and Hawkes, 2009).

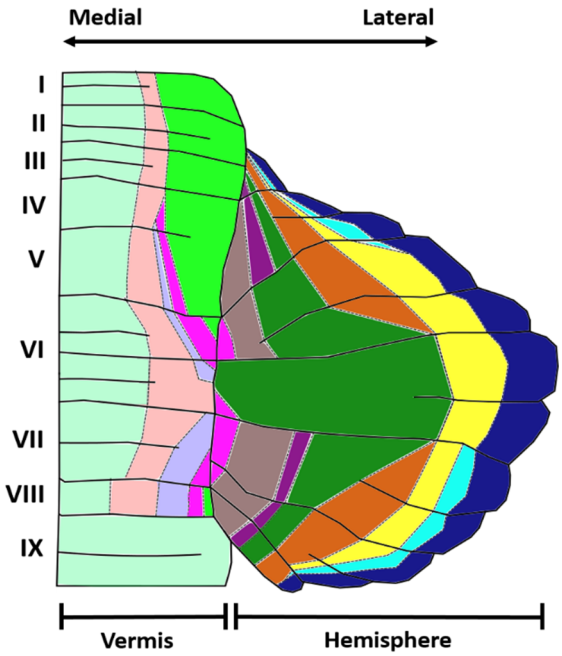


Fig. 1.4: Organisation of cerebellar longitudinal zones in rats. Flattened view of the cerebellar vermis and one hemisphere with flocculus/ paraflocculus and Lobule X omitted. From medial to lateral, longitudinal zones: A (), AX (), X (), A2 (), B (), C1 (), CX (), C2 (), C3 (), D1 (), D0 (), D2 (). Adapted from Sugihara and Shinoda (2004); Voogd and Ruigrok (2004); Apps and Hawkes (2009).

Largely through electrophysiological work it has been recognised that functional zones can be further subdivided into microzones (e.g. Andersson and Oscarsson 1978; Ekerot and Larson, 1978; Oscarsson, 1979; Garwicz and Ekerot, 1994; Hesslow, 1994a, b; Yeo and Hesslow, 1998). As subdivisions of functional zones, microzones are long narrow strips oriented transversely to the long axis of the folium. Microzones are ~100-300 μm wide whereas zones are ~1-2 mm wide. The best studied regions are the C1 and C3 zones of lobule V and these may serve as generally representative of wider cerebellar territories. Purkinje cells in these well-studied microzones of C1 and C3 receive input from olivary neurons with a shared somatosensory receptive field and, via its output to the cerebellar nuclei, each microzone exerts control over a specific muscle or muscle group that has a functional relationship with its olivary receptive field.

The compartmental organization of projections from IO to cerebellar cortex to cerebellar nuclei is also preserved in the inhibitory nucleo-olivary projection. The inhibitory nucleo-olivary neurons that receive input from Purkinje cells of a particular zone project to the olivary sub-nucleus that also gives rise to the CFs that define that cerebellar zone (Ruigrok, 1997). This olivo-cortico-nucleo-olivary loop connection creates a cerebellar module (Pjipers et al, 2006) and within it is a sub-loop where CF collaterals innervate a nuclear zone that corresponds to the cortical zone innervated by the ascending CF fibres (Groenewegen and Voogd, 1977; Andersson and Oscarsson, 1978; Voogd and Ruigrok, 2000; Sugihara and Shinoda, 2007). Finally, the dendritic

branching of MLIs and their CF input also appear to be restricted to particular zones, in correspondence with the zonal identity of their PC targets (Ekerot and Jorntell, 2001; Gao et al, 2001; Jorntell and Ekerot, 2003). The ascending dendritic branch of GoCs is has also been shown to be restricted to a specific parasagittal zone in mice (Sillitoe et al, 2008). These connection properties suggest that the longitudinal zones and microzones are fundamental information processing modules within the cerebellar cortex (analogous to cortical columns in the cerebral cortex) such that distinct and non-overlapping processing can take place within each module.

In addition to the essentially connectionist principle of cerebellar cortical organisation described above, there is an additional organisational principle at the molecular level. Parasagittal stripes throughout much of the cerebellar cortex can be defined by the presence or absence of expression of various molecular markers that include phosopholipase Cβ4, excitatory amino-acid transporter 4 (EAAT4), heat shock protein 25 (HSP25) and many others (Apps and Hawkes, 2009). The best studied of these markers is Zebrin II (Brochu et al, 1990), which was later demonstrated to be Aldolase C, a key enzyme in the glycolysis metabolic pathway. The expression of Zebrin II/ Aldolase C can be used to separate the cerebellar cortex into rostrocaudally extensive stripes of PCs that are alternately Zebrin II positive and Zebrin II negative (Zebrin II^+ and Zebrin II^-, respectively). As with the longitudinal zones, the pattern of $Zebrin^{+/-}$ PCs is symmetrical across the midline, it is highly conserved within species and the general pattern is largely conserved across species. The stripe organisation of Zebrin II is not universal across the entire cerebellar cortex. Some regions, including the vermis, paraflocculus and flocculus of lobule X and parts of Lobule VI are uniformly Zebrin II^+ (Apps and Hawkes, 2009). However other molecular markers, such as HSP25, reveal different patterns of stripes that subdivide some of the uniformly Zebrin II^+ regions into further distinct stripes (Armstrong et al, 2000). There is a general congruence between the borders of Zebrin II^+ and Zebrin II^- stripes and longitudinal functional zones within the cerebellar cortex suggesting some relationship between the two (Sugihara and Shinoda, 2004; Voogd and Ruigrok, 2004; Pjipers et al, 2006; Sugihara and Shinoda, 2007).

In summary, whilst the cerebellar cortex has a largely identical cytoarchitecture it is arranged functionally into a large number microzones defined by their connectivity. Ito (2006) estimates there could be as many as 5000 microzones in the human cerebellum.

1.1.6 Neuromodulators and neuropeptides: A significant but little understood third afferent system in the cerebellum

Owing to their conspicuous appearance, numerical significance and central role in influential theoretical accounts of cerebellar function (Marr, 1969; Albus, 1971; Ito, 1972; Gilbert, 1974 and others) the glutamatergic MF and CF cerebellar afferents have been the main focus of investigations over the last four to five decades. However there are important other cerebellar afferent fibres that can be loosely differentiated from MFs and CFs by their beaded appearance. These beaded fibres have fibre swellings or varicosities along their length and these are thought to be sites of neurotransmitter/neuromodulator release (Ito, 2006; Ito, 2009). These afferent types can be split into three main groups according to the agent released: (i) acetylcholine (ii) neuropeptides (ii) the monoamines, including 5-HT, dopamine (DA), histamine and noradrenaline (NA) (see Ito, 1984; Ito, 2009; Schweighofer et al, 2004). The majority of receptors for these neurotransmitters/neuromodulators are metabotropic with the exception of the 5-HT_3 receptor and the nictonic acetylcholine receptor.

Acetycholine:

Acetylcholine fibres innervate the cerebellar cortex and nuclei as a diffuse projection of beaded fibres (Barmack et al, 1992a, b) and some are reported to terminate as mossy fibre rosettes in the GCL of specific cortical lobules (Barmack et al, 1992a, b). Both the ligand-gated, cation channel, nicotinic cholinergic receptor and the metabotropic, G-protein coupled, muscarinic cholinergic receptor have been found in the cerebellum of various species (reviewed in Jaarsma et al, 1997). Muscarinic receptor activation enhances PC responsiveness to glutamate application in anaesthetised rats (Andre et al, 1993) and cholinergic afferents have an excitatory effect on cerebellar nuclear neuron activity (Vitale et al, 2016). Muscarinic receptor activation inhibits plasticity mechanisms at the PF-PC synapse via activation of endocannabinoid activation (Rinaldo and Hansel, 2013) and acetylcholine signalling mediated by the nicotinic receptor in the GCL may modulate some forms of plasticity (Prestori et al, 2013). The role of acetycholine signalling in cerebellar function has been studied in far more depth then than other neuromodulators (Jaarsma et al, 1997) but analysis of this literature is beyond the scope of this study.

Neuropeptides:

Peptidergic beaded afferents to the cerebellum include those that release angiotensin II, Dynorphin, Leu-enkephalin (Leu-ENK), Met-enkephalin (Met-ENK) and orexin (Ito, 2009). In addition to release by these afferent fibre types, neuroactive peptides are also coexpressed and released by CFs (e.g. corticotropin-releasing factor - CRF) and MFs (e.g. CRF, leu-ENK, met-ENK and substance P) and several neuropeptides (e.g. cerebellin, motilin and galanin) are co-expressed in some cerebellar cortical neuronal types including PCs, GoCs and Lugaro cells. A total of 22 neuroactive peptides have been located in the cerebellum (see Ito, 2009 for a review). A few unrelated studies have examined some aspects of the functions of these peptides but there is no coherent body of research that has yet fully revealed the importance of neuropeptide signalling in the cerebellum.

Dopamine:

Traditionally there was thought to be no dopaminergic innervation of the cerebellum, but a dopaminergic input originating in the ventral tegmental area (VTA) and projecting to the GCL and PCL has been reported (Ikai et al, 1992). Barili et al (2000) observed very low levels of IR for dopamine receptors D1-D5 in the the GCL and PCs and a recent study examining IR of the dopamine membrane transporter, a marker for dopaminergic fibres, found a high density of dopaminergic afferents in the upper GCL just below the PCL in the primate cerebellum (Melchitzky and Lewis, 2000). Very little experimental work has examined the physiological effects of dopamine in the cerebellar cortex or dopaminergic involvement in cerebellar dependent plasticity or learning processes.

Histamine:

Histaminergic afferents to the cerebellum originate in the tuberomammillary nucleus in the hypothalamus (Panula et al, 1989; 1993). Afferent fibres are present in the ML and GCL and cerebellar nuclei and the presence of H1, H2 and H3 in the cerebellum has been demonstrated (Pollard et al, 1993; Arrang et al, 1995; Vizuete et al, 1997). *In vitro* recordings have demonstrated an excitatory PC response evoked by histamine application that is mediated by H2 receptors. A recent literature has begun to reveal distinct and complex functions of histaminergic signalling in the consolidation of emotional memory tasks and dependent upon H1 and H2 receptor activation in the cerebellum (Gianlorenco et al, 2011; 2012; 2013; 2015).

Noadrenaline and Serotonin:

The two most prominent neuromodulatory inputs to the cerebellum are the noradrenergic and serotonergic afferents (Ito, 1984). Relative to the other aminergic afferents acetylcholine, dopamine and histamine and to the neuropeptides there is a greater body of research concerning the anatomical distribution of noradrenergic and serotonergic afferents and the related receptor and transporter signalling proteins. The physiological effects of noradrenergic and serotonergic signalling in the cerebellum and the role of noradrenaline and 5-HT in cerebellar-dependent behaviours have also been studied in more detail. The specific role of 5-HT and noradrenaline in cerebellar functions are the focus of this thesis and a detailed review of these systems will be found in later in this chapter.

1.2 The cerebellum as a structure for motor control and learning

1.2.1 The cerebellum and motor function

The earliest understanding of the cerebellum and its functions came through examination of the sequela of experimentally induced cerebellar lesions in animals (Rolando; Fluorens; Dalton: Luciani) and clinical examination of human cerebellar lesion patients (Gowers; Babinski; Holmes; reviewed in Fine et al, 2002). A common outcome of lesions in both animals and humans is movement dysfunction. The major sign of cerebellar damage is ataxia, where the coordination of movement timing and magnitude is compromised yet the ability to initiate and execute movements is largely spared (Holmes, 1939; Fine et al, 2002).

1.2.2 The universal cerebellar transform and motor learning

Based on the consistent microcircuitry that is a defining feature of the cerebellar cortex many of the most influential theories of cerebellar function propose that the cerebellar cortex implements a universal form of information processing across its surface. The theories that have been most influential and that have inspired most experimental research can be broadly defined as learning theories: theories that propose the cerebellar microcircuit is ideally suited to the learning of associations or procedures. In particular the emphasis of the majority of early theories was on the how the cerebellum could learn motor skills.

The first of these theories was by Marr (1969) who suggested that the PC must transmit some type of motor command. Marr assumed that PC activation (and in turn the cerebellar motor command) was predominantly generated by mossy fibre signalling and suggested that MFs originating from a range of precerebellar nuclei transmitted information related to the current ‘context’ of movement selection. Central to the theory was the extensive divergence of MF inputs onto individual PCs via the parallel fibres. Marr proposed that the granule cell layer acts as a pattern separator, amplifying the differences in incoming MF activity patterns so that even highly similar patterns would arrive at the PC as distinguishable inputs. Marr proposed that each olivary neuron represents an ‘elemental movement’ e.g. an isolated limb or finger movement and that

this olivary signal is driven by motor regions of the cerebral cortex. Each firing of the olivary neuron and its resultant complex spike would selectively potentiate the currently firing PFs such that, after repeated pairings of CF and PF activity, the cerebellar cortex could learn to select and coordinate movements under specific environmental control (as represented by a specific PF pattern) without further cerebral cortex involvement.

Marr's theory was soon followed by a proposal from Albus (1971) that had some similarities but two important differences. First, he suggested that the olivary neuron provides a motor error signal to the PC and second, he suggested that the associative change in PF synapses would be a decrease in efficacy, not an increase, leading to reduction in PC firing rates in response to a particular, recognised patterns of PF activity. The combination of the critical aspects of each of these model has come to be referred to collectively as the Marr-Albus model.

Ito (1972) took the main elements of the Marr-Albus formulation and applied them to a specific cerebellar system: the vestibulocerebellum and a particular function of that region: adaptive control of the vestibulo-ocular reflex (VOR) in the face of external or internal disturbances to its performance. The VOR is a reflex eye movement in response to stimulation of the vestibular system that helps stabilize the retinal image during head movements. The flocculus receives primary vestibular afferents in the form of MF inputs and the PCs of the flocculus (amongst other regions of the vestibulocerebellum) project to the vestibular nuclei. If the VOR is not properly calibrated there is slippage of the visual image on the retina. Ito (1972) postulated that climbing fibres projecting to the flocculus transmit a signal related to this retinal slip and that this error signal modifies the output of the flocculus over trials. This changed floccular output will modulate the vestibular nuclei output to its oculomotor targets, thus giving a recalibrated VOR. This specific application of the Marr-Albus model has received a wide range of experimental support for many of its central tenets (Ito, 1982; Boyden et al, 2004). For example, Robinson (1976) found that bilateral removal of the flocculus prevents adaptive modification of the VOR. Ito et al (1981) observed the same effects with unilateral lesions and Ito et al (1980) prevented adaptation of the VOR following kainic acid induced lesions of the flocculus. Analysis of the closely related optokinetic reflex (Nagao, 1983, Shutoh et al 2006; Okamoto et al, 2011a, b) also gave an early indication of the cerebellar involvement in its performance and adaptation.

An aspect of note in Ito's theory is the nature of the CF signal. Marr (1969) required the input of the cerebral cortex for the CF signal and Albus (1971) proposed that the CF error signal reflected the difference between the desired outcome and the actual outcome, derived from sensory feedback. Ito's model does not require cortical control or an internal representation of desired movement outcomes in a sensory 'language' but uses a sensory signal directed to a specific region of the cerebellum which is related to the movement controlled by that region and occurs as a consequence of an 'error' in the specific movement category.

The Marr-Albus model has continued to be highly influential in theoretical accounts of cerebellar function and with some adaptation to accommodate emerging data its central tenets remain a good description of cerebellar function (Houk et al, 1996; Boyden et al, 2004; Dean et al, 2010; Dean and Porrill, 2014). Following Ito (1972), other researchers have applied and extended the model describe a range of cerebellum-dependent behaviours (Fujita, 1982; Moore et al, 1989; Kawato and Gomi, 1992; Dean et al, 2004; Lepora et al, 2010; Dean et al, 2013).

1.2.3 Classical conditioning of reflexes and their reliance on the cerebellum

The model proposed by Ito (1972) was especially notable because it introduced the possibility that the Marr-Albus model could be applied to the adaptation and calibration of simple reflex actions. The importance of the cerebellum in the control of reflex movements was established in a sphere of research especially involving the Russian Pavlovian behaviourists and largely separate from the early work on the cerebellar involvement in voluntary movement (see Welsh and Harvey, 1992; Bloedel and Bracha, 1995 for reviews). In particular, they examined the role of the cerebellum in the adaptation of reflex movements using classical, also known as Pavlovian, conditioning methods. Classical conditioning requires a pre-existing reflex, for which there is a known eliciting stimulus, the unconditional stimulus (US) that reliably and unconditionally drives an identified reflexive response, the unconditional response (UR). If a neutral stimulus, the conditional stimulus (CS) is reliably and predictably paired with the US it comes to elicit a reflex response, the conditional response (CR) with properties often similar to those of the UR.

In one of the first studies of cerebellar involvement in classical conditioning, Popov (1929) (reviewed in Bloedel and Bracha, 1995) observed a disruption of classical conditioning of leg flexion after bilateral or unilateral cerebellectomy or after removal of the vermis. Later studies by Karamyan (1959) and Fanardjian (1961) (reviewed in Bloedel and Bracha, 1995) also reported disruptions of classical conditioning of leg flexion following cerebellar lesions. Researchers outside Russia also observed effects of cerebellar lesions on leg flexion conditioning. Unilateral lesions of the dentate nucleus and/or the lateral interpositus nucleus region abolished conditioning (Chambers and Sprague, 1955a; b) whereas paravermal cortical lesions (Chambers and Sprague (1955a, b; Yu, 1972) or cooling (Yu, 1972) led to hyper-excitability of the UR. Lesions of cerebellar efferent targets in the red nucleus also abolished conditioned leg flexion (Smith, 1970).

1.2.4 Nictitating membrane and eye-blink response conditioning: Key models in understanding cerebellar learning

The role of the cerebellum in the control and conditioning of another pair of reflexes has been intensively studied. This pair is the nictitating membrane response (NMR) in rabbits (McCormick and Thomspon, 1984; Yeo et al, 1985a, b, c; Hesslow and Yeo, 2002) and the functionally related eyelid blink response (EBR) (Garcia and Mauk, 1999; Garcia et al, 1999; Gruart et al, 1997). The EBR and NMR share some similarities but differ in certain key parameters including their final neural control pathways (van Ham and Yeo, 1996; Longley and Yeo, 2014). The NM in the rabbit is a translucent cartilaginous membrane, sometimes referred to as the third eyelid that sweeps horizontally across the eye to cover and protect the eyeball.

Classical conditioning of the NMR is an example of associative learning of a behaviour which is relatively free from confounding non-associative mechanisms such as sensitization and pseudo-conditioning (Gormezano et al, 1962; Schneiderman et al, 1962; Thompson, 1976). Despite differences in their neural control circuitry, the protocol for classical conditioning of the NMR and EBR in rabbits is the same because the typical corneal airpuff or periocular stimulation US used elicits a coordinated response in both systems (Attwell et al, 2002a). The basic protocol is as follows: a tone, light or tactile/ electrical skin stimulation conditional stimulus (CS) is paired with an airpuff to the cornea, or periocular region or electrical stimulation of the periocular area, as an unconditional stimulus (US). The US elicits the NMR/EBR on initial

presentations but repeated pairing of the CS and US leads to development of a well-timed CR to the previously neutral CS. Two forms of classical conditioning can be distinguished on the basis of the relative timing of the CS and US presentation. In delay conditioning the CS and US overlap whilst in trace conditioning the presentation of the CS is terminated before the presentation of the US, leaving a 'trace period' with no stimulus presentation. Unless explicitly stated, classical NMR or EBR conditioning studies discussed within this thesis will refer to delay conditioning protocols. Although both NM and EB responses present as a coordinated compound UR and subsequently as a compound CR with the same conditioning protocol, work in the host laboratory has focused specifically on classical conditioning of the NMR. The NMR has a low baseline response rate compared to the EBR allowing for easy measurement of associative changes (Gormezano et al, 1962; Schneiderman et al, 1962, Thompson, 1976; Hesslow and Yeo, 2002) and because the absence of voluntary NM responses appears to relate to an absence of motor cortical drive to NMR control circuits, there a better opportunity to isolate the cerebellar contribution to the changes that underlie these forms of associative learning (Longley and Yeo, 2014)

1.2.5 Dependence of nictitating membrane response conditioning on the cerebellum

A set of early studies by Oakley and Russell (1972, 1975 and 1977) showed that acquisition and retention of NMR conditioning in rabbits is intact after bilateral decortication, demonstrating the absence of essential cerebral cortical control of NMR conditioning. Later, Mauk and Thompson (1987) ruled out the involvement of the entire forebrain by showing intact retention of NMR conditioning following decerebration at a level rostral to the red nucleus.

Thompson and colleagues reported a series of studies suggesting that the cerebellum is a critical structure for classical conditioning of the NMR. McCormick et al (1981 and 1982a) reported abolition of a previously learned CR after large ablations of the ipsilateral cerebellum. The ablations had no effect on the UR and unilateral lesions prevented the acquisition of conditioned NMR/EBR in the ipsilateral but not contralateral CR (Lincoln et al 1982). Lesions restricted to the cerebellar nuclei, the targets of the cerebellar cortical output and the only efferent pathway of non-vestibular cerebellum were as effective in abolishing conditioned responses as were larger lesions. Clark et al (1984) and McCormick and Thompson (1984) made lesions of the

dentate-interpositus nuclei and observed a loss of acquired CRs with no effects upon the UR and a CR that could be retrained on the side contralateral to the lesion. Additionally, McCormick and Thompson (1984) found that lesions of the adjacent lateral dentate region had no effect on CR performance. Efferents of the dentate and interpositus nuclei exit the cerebellum through the superior cerebellar peduncle and lesions of this fibre bundle also abolish pre-established NMR conditioning (Lavond et al, 1981; Desmond and Moore, 1982 and McCormick et al, 1982b) and prevent reacquisition (Desmond and Moore, 1982). Lesions to the red nucleus (Rosenfield and Moore, 1983), a major target of the interpositus and dentate nuclear efferents, and lesions of the tracts connecting the cerebellum to the RN also abolish pre-established NM CRs (Rosenfield and Moore 1985). Lesions in the region of the rubrobulbar tract, that contains projections from RN to the accessory abducens nucleus, whose motoneurons control the retractor bulbi muscle mainly responsible for the NMR, also abolish NM CRs (Rosenfield et al, 1985).

Yeo et al (1985a) extended these findings by making discrete lesions of the different cerebellar nuclei and found that only those lesions that included the anterior interpositus nucleus (AIP) abolished pre-established CRs to both visual and auditory CSs and prevented their reacquisition.

1.2.6 Requirement of the cerebellar cortex and nuclei in NMR conditioning

In contrast to the reported effects of restricted lesions of the cerebellar nuclei, McCormick and Thompson (1984) claimed that lesions encompassing different regions of the cortex failed to abolish pre-established CRs, though they noted a disruption of CR timing in animals where lesions “included the ansiform lobule”. Woodruff-Pak et al (1985) and Lavond et al (1987) later reported impairments or abolition of CRs with cortical lesions but they were able to retrain CRs in trace and delay eye-blink conditioning, respectively. However, Yeo et al (1984; 1985b) found that lesions of the cerebellar cortex did abolish CRs and prevented reacquisition. Crucially, Yeo et al (1985b) took a systematic approach to localisation of the critical lesion sites and after examining the consequences of larger lesions they made more restricted lesions of hemispheral lobule VI (HVI) and found that only those lesions that included the base of the lobule led to complete abolition of the CR and prevented reacquisition. Lesions restricted to dorsal regions of this lobule were less effective or they led to loss of CRs which were recovered on further training. This finding is consistent with the suggestion

that previous studies saw no loss of CRs, or only a temporary loss, because the lesions did not include HVI or only included only its more accessible dorsal regions.

Clearly, the suggested importance of cerebellar cortex in NMR conditioning is more consistent with the Marr-Albus model of cerebellar learning. By observing cell degeneration in the IO, the same authors (Yeo et al, 1985b) found that the medial dorsal accessory olive (mDAO) which has is known to receive tactile information from the face via the trigeminal nerve (van Ham and Yeo, 1992) provides CF input to Lobule HVI. Using the terminology of the Marr-Albus model the CF projection from the mDAO to lobule HVI could transmit the 'teaching' signal. In the third paper of its series, Yeo et al (1985c) showed the AIP is a major target of lobule HVI PC efferents, consistent with the accompanying behavioural studies of AIP and HVI lesions (Yeo et al 1985a, b) and in agreement with subsequent anatomical work (Gonzalez-Joekes and Schreurs, 2012) It was shown that Lobule HVI receives a set of afferents from the mDAO, and another set of afferents from the basilar pontine nuclei and NRTP (Yeo et al 1985c). Both the pontine nuclei and NRTP are sources of MFs to the cerebellar cortex and the pontine nuclei receive visual and auditory information via the superior and inferior colliculi respectively and hence could be the substrate for CS related information that eventually elicits the motor response (CR). Thus there is a convergence of mossy and climbing fibre inputs, each conveying the appropriate CS and US-related information, to lobule HVI. This convergence satisfies a requirement that could support a Marr-Albus learning process in NMR/EBR conditioning (Hesslow and Yeo, 2002).

This idea was developed and made explicit by Hesslow and Yeo (2002) who proposed the cerebellar cortical conditioning (CCC) model, an implementation of the Marr-Albus model for NMR/EBR conditioning. The US related information provided by the CFs and originating in the mDAO and CS information from MFs originating in the pontine nuclei converges on PCs in lobule HVI. The role of mossy fibres in providing the CS-related information was strongly indicated in a study by Hesslow et al (1999). EBR conditioning was established in decerebrate ferrets with electrical forelimb stimulation as the CS. After CRs were established, substitution of middle cerebellar peduncle (MCP) stimulation as the CS evoked CRs with no additional training. Because the MCP, contains the ponto-cerebellar MFs, this finding strongly supported the proposition that MFs convey the CS signal. The CCC model predicts that the US and CS signals converge upon PCs and repeated pairing produces a conditioned pause in PC activity

is response to the specific pattern of pontine MF activity. The pause in PC activity disinhibits target CN neurons in the AIP and evokes a conditioned NMR/EBR.

1.2.7 Conditioned responses are controlled by activity of specific Purkinje cells in Lobule HVI

Studies that have combined classical conditioning, using either conventional presentation of CS and US at the periphery or using analogous stimulation in the CNS, with electrophysiological recording from Purkinje cells have provided support for predictions of the CCC model and confirmed the localisation of critical structures in NMR/EBR conditioning. Hesslow (1994a, b) discovered two eyeblink control regions in lobule HVI in decerebrate cats that showed short latency CF responses (~8 ms) evoked by periocular stimulation within a small receptive field. Electrical stimulation of these cortical eyeblink control regions evoked an EMG response in the orbicularis oculi muscle (the agonist muscle that controls the EBR) at train offset. Such stimulation also inhibited production of ongoing CRs to simultaneous peripheral CS stimulation but had only a weak effect on ongoing URs. Taken together, these results indicating that the cortical eyeblink control zone has specific control of the CR. These identified regions are small and constitute only a very small part of lobule HVI. They closely correspond to the region of lobule HVI that Yeo et al (1985a, b and c) identified as critical for rabbit NMR conditioning. Mostofi et al (2010) made a detailed electrophysiological analysis of eyeblink control regions in the rabbit and confirmed that they are similar to those characterised by Hesslow for cat (Hesslow 1994a, b). They identified PCs in a region at the base of the medial wall of lobule HVI that responded to ipsilateral periocular stimulation with a short latency CF response. This location is consistent with the location of regions of HVI cortex essential for NMR conditioning previously identified (Yeo et al, 1985b). Mostofi et al (2010) further confirmed that these PCs controlled conditioned NMR performance by using small infusions of CNQX, an antagonist of AMPA/ Kainate type glutamate receptors, restricted to this region which reduced or completely abolished CRs.

Heiney et al (2014) identified similar regions in awake head-fixed mice. Stimulation of these regions elicited EBRs at stimulation offset. Additionally, they showed that inhibition of PC activity in this region by optogenetic activation of local MLIs, which inhibit local PC activity, led to an EBR that was time-locked not to the offset of the stimulation but to the onset of the stimulation and so supporting the CCC model

prediction that a pause in PC activity elicits the conditioned NMR/EBR. Halverson et al (2015) recorded from PCs in lobule HVI in awake rabbits that they had defined as 'eyelid PCs' and saw a systematic relationship between the timing of the PC pause response and the ISI to which the animal was trained and between the magnitude of the pause and the presence or absence of a CR. Additionally, in mice, ten Brinke et al (2015) showed that PCs they classified as eyelid-related showed a CS-related simple spike suppression, the size of which correlated with CR occurrence and amplitude on a trial by trial basis. There was no statistical relationship observed between the group simple spike magnitude and any aspect of the CR, however this may be a result of the heterogeneous nature of the cell population samples. They used a 60 ms complex spike latency to an airpuff US as the grouping variable, so it is highly unlikely that this loose criterion was sufficient to allow sampling of eye-blink controlling PCs as defined in the earlier studies by Hesslow (1994a, b) and Mostofi et al (2010). Similarly weak criteria were used in another recent study of mouse lobule HVI by Ohmae and Medina (2015) and the observations were similar to those of ten Brinke et al (2015).

In contrast to these recent electrophysiological studies on rabbit (Halverson et al 2015) and mice (ten Brinke et al 2015; Ohmae and Medina, 2015), Jirenhed et al (2007) recorded from PCs unambiguously identified as eye-blink controlling in decerebrate ferrets. PC activity was recorded over time periods long enough to track activity during combinations of learning, extinction and reacquisition. In all the PCs followed through conditioning, cell firing was suppressed during the ISI, the latency of the suppression varied according to the ISI and the maximum suppression was well timed to precede the presentation of the US. Furthermore, unpaired US and CS presentations or CS presentation alone, which are known to cause behavioural extinction, here caused an extinction of the PC firing suppression. The PC firing suppression was reinstated when paired stimulus presentations were reintroduced. These PC suppression responses parallel many of the features of classical conditioning of the NMR/EBR and are consistent with the suggestions that the CS-US association is encoded in specific PCs in an eyeblink control region within Lobule HVI and that output from this region exerts control over the CR.

1.2.8 Classical conditioning of the nictitating membrane or eye-blink response: differentiating acquisition and performance

Whilst lesion and tracing studies were successful in identifying which olivo-cortico-nuclear compartment is critical for acquisition of NMR conditioning they cannot distinguish between the mDOA, lobule HVI and AIP in identifying the critical site of memory acquisition and storage because the effects of lesions on performance cannot be dissociated from the effects on acquisition and retention. However, localized drug infusions that reversibly inactivate or disrupt activity in specific structures can overcome this disadvantage by allowing short-term disruption of processing during learning without a long-term effect on performance. The first reversible inactivation study found normal NMR conditioning to a tone CS after an infusion of lidocaine into the AIP during acquisition (Welsh and Harvey, 1991). However this result was confounded by the double training protocol and its potential for transfer effects (Longley and Yeo, 2014) and subsequent studies did report clear disruptions of learning following pre-training infusion of the $GABA_A$ agonist muscimol into the AIP (Krupa et al, 1993, Hardiman et al, 1996, Ramnani and Yeo, 1996 and Yeo et al, 1997).

Pre-training olivary lidocaine infusions also prevent CR acquisition (Welsh and Harvey, 1998) and, crucially for the proposition that NMR conditioning depends upon a cerebellar cortical mechanism, pre-training infusions of CNQX can be shown to be restricted to lobule HVI and they prevented CR acquisition (Attwell et al, 1999; 2001). Finally, Krupa and Thompson (1995) found that reversible inactivation of the SCP, the fibre tract that conveys cerebellar output in NMR conditioning did not affect acquisition. In summary, pretraining inactivation of a node within the olivo-cortico-nuclear compartment can prevent acquisition, so one interpretation may be that critical acquisition mechanisms and the sites of memory storage are distributed across the olivary, cortical and nuclear levels.

However, an alternative interpretation is possible. The cerebellar nuclei contain a set of inhibitory neurons that project to the inferior olive (Hesslow, 1986; Andersson et al, 1988; Nelson and Mugnaini, 1988 and Svensson et al, 2006). The olivo-cortico-nuclear compartment influenced by these inhibitory neurons is the compartment within which they have their nuclear location and the entire circuit forms an Olivo-Cortico-Nucleo-Olivary loop (O-C-N-O loop). Disruption of activity at any of level in one of these compartments has been shown to have a significant effect on activity at the other two levels. For example inactivation or increased activation of the olive leads to increases

or decreases of PC simple spike frequency, respectively (Rawson and Tilokskulchai, 1981; Montarolo et al, 1982; Cerminara and Rawson, 2004). Disrupting the inhibitory nucleo-olivary pathway affects PC excitability (Bengtsson et al, 2004; Svensson et al, 2006) and activity in the cerebellar nuclei (Nilaweera et al, 2002 and Zbarska et al, 2008). So reversible inactivations of the different nodes in the O-C-N-O loop are likely to disrupt processing at all nodes around the loop. With these compartmental changes of activity following lesions and pre-training reversible inactivations, interpretation of their effects upon conditioning must be limited. We can conclude only that the cerebellum is required for the production of CRs in classical conditioning of the NMR/EBR and normal functioning of the O-C-N-O loop is required for its acquisition.

Another approach is required in order unambiguously to determine how the memory is stored - whether it is distributed throughout the cerebellar circuitry, localised specifically to the cerebellar nuclei or localised specifically to the cerebellar cortex, as predicted by the Marr-Albus and CCC models. Attwell et al (2002b) made *post-training* infusions of muscimol in lobule HVI or AIP in order to disrupt potential consolidation mechanisms crucial to long term memory storage. It was argued that the localisation of consolidation processes is a more direct test of the localisation of memory storage and these processes might be less influenced by general activity changes around the OCNO loop. Muscimol infusions directed to Lobule HVI, restricted to the cortex (Attwell et al, 1999) and made shortly after each of four, daily training session held off the acquisition of the NM CR over the four sessions whereas NM CRs developed normally in animals that received post-training AIP infusions. These results suggested that consolidation of NMR/EBR conditioning is mediated by mechanisms within the cerebellar cortex and thus learning related plasticity is localised to the cerebellar cortex, as predicted by Marr (1969) and Albus (1971). However, another interpretation was possible. Muscimol infusions to the cerebellar cortex depress PC activity which disinhibits their cerebellar nuclear target neurons. In contrast, muscimol infusions directly to the cerebellar nuclei hyperpolarize them and reduce their activity (Aksenov et al, 2004). These different effects upon nuclear excitability changes raised the possibility that consolidation may, in fact, be occurring in the cerebellar nuclei but that it is particularly susceptible to disruption by hyper-excitability, as would be found following cortical muscimol infusions. This possibility was tested by Kellett et al (2010) who made simultaneous muscimol infusions in lobule HVI and AIP which would have decreased activity both in the cerebellar nuclei and in the cortex. When such double infusions were made in the same post-training as before, consolidation was again disrupted, ruling out an excitation-sensitive nuclear consolidation mechanism and strongly supporting the

suggestion that consolidation processes are intracortical and so confirming a cortical memory storage mechanism. Post-training cortical infusions delayed by 5 or 45 minutes prevented consolidation whilst infusions delayed by 90 minutes were ineffective, indicating that these muscimol-sensitive, cortical consolidation process are complete within two hours after training (Cooke et al, 2004).

1.2.9 The cerebellum and motor learning: Summary

The cerebellum is known to be critical for the coordination of voluntary movements and the performance of certain reflex behaviours. A set of early and influential theoretical accounts of cerebellar processing predicted a critical role for the cerebellum in motor learning (Marr, 1969; Albus, 1971; Ito, 1972 and Gilbert, 1974). Later experimental work examining classical conditioning of the NMR in rabbits and classical conditioning of the functionally related EBR in other species demonstrated the critical role of the cerebellum in associative learning in this task. The cerebellar cortex has been identified as a site of consolidation processes and memory storage using localised post-training drug infusions. A site within lobule HVI has been identified (Kellett et al, 2010 and Longley and Yeo, 2014) for this memory storage and electrophysiological studies have shown that a suppression of PC activity driven by an associative mechanism that depends upon coincident CF and MF activation in this critical region may be the change that underpins the conditioned response (Jirenhed et al, 2007 and 2013).

1.3 Monoamines: Anatomy, physiology and theoretical perspectives

In addition to the glutamatergic CF and MF afferents, the cerebellum also receives a range of afferent fibres that release either a monoamine, acetylcholine or a neuropeptide. The focus here will be on the two largest, non-glutamatergic afferent populations, the monoaminergic noradrenaline (NA) and serotonin (5-HT) systems. Noradrenergic and serotonergic afferents and their corresponding receptors are present throughout the cerebellar cortex and cerebellar nuclei. There are proposed functions for both of these neuromodulatory systems in learning and memory formation in other regions of the brain and so there is good reason to suspect that they may be involved in learning processes in the cerebellum. In this chapter I will summarise the current understanding of the physiological actions and functional role of these two systems in the cerebellum and in learning and memory processes.

1.3.1 Noradrenaline: General anatomy and receptor pharmacology

Cerebellar noradrenergic afferents enter the cerebellum via the SCP and the main source of noradrenergic afferents to the cerebellum is the locus coeruleus (LC) (Olson and Fuxe, 1971; Ungerstedt, 1971; Pickel et al, 1974). The locus coeruleus is found bilaterally just ventral to the fourth ventricle in the rostral pons and it is the principal site of noradrenaline synthesis in the brain. It is the source of approximately 50% of noradrenergic afferents in the CNS and all projection neurons within the LC are noradrenergic. There are sets of smaller, more loosely distributed noradrenergic nuclei in the pons and medulla; these include the subcoeruleus immediately ventral to the LC and the noradrenergic cell groups A1-A7 (Robertson et al, 2013). Though smaller in number than those from the LC, a significant number of cerebellar afferents originate in the subcoeruleus, A1, A2, A5 and A7 (Chu and Bloom, 1971; Pasquier et al, 1980; Robertson et al, 2013). Noradrenergic afferents target all three layers of the cerebellar cortex and the cerebellar nuclei (Hökfelt and Fuxe, 1969; Bloom et al, 1971; Segal et al, 1973; Mugnaini and Dahl, 1975; Felten et al, 1986; Di Mauro et al, 2003). A more detailed review of the distribution of noradrenergic afferents in the cerebellum can be found in Chapter 3.

In addition to its cerebellar projections, the LC projects to terminal fields covering a large portion of the brain including most functional divisions of the cerebral cortex (Aston-Jones and Cohen, 2005), olfactory bulb, hippocampus, thalamus, hypothalamus, amygdala, bed nucleus of the stria terminalis, superior colliculi, brainstem and spinal cord (Robertson et al, 2013 and Simpson and Lin, 2007).

There are a nine noradrenaline receptor types (adrenoceptors or ARs) expressed throughout the brain and all are G-protein coupled metabotropic receptors. There are two families: α- and β-adrenoceptors. α-adrenoceptors are further divided into two types, α_1 and α_2-adrenoceptors and further divided into subtypes: α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B} and α_{2C}. β-adrenoceptors are divided into three types, β_1, β_2 and β_3-adrenoceptors.

All three β-adrenoceptor subtypes are coupled to Gs proteins. The Gs protein activates adenylate cyclase that stimulates cyclic adenosine monophosphate (cAMP) production which in turn activates cAMP-activated protein kinase A (PKA) and then cAMP response element binding protein (CREB). This pathway is of particular interest in learning and memory research as CREB is a transcription factor and thus can directly increase or decrease transcription of specific genes that can ultimately result in an increase or decrease of protein synthesis. Such processes are widely regarded as importantly part of the mechanisms underlying long-term memory formation (Fig 1.5).

α_1-adrenoceptors are Gq protein coupled, the activation of which can ultimately stimulate the production of inositol triphosphate (IP3) which stimulates an increase of cytosolic calcium levels through release from intracellular stores. It can also simultaneously lead to the production of diacylglycerol (DAG) which activates protein kinase C (PKC) which is known to phosphorylate a large number of membrane bound channels and pumps (Fig. 1.5).

In comparison to the other types that are expressed postsynaptically, the α_2-adrenoceptors are expressed both postsynaptically and presynaptically, on noradrenergic neurons and their fibres, and so it is the only one of the NA receptor subgroups that can act as an autoreceptor (although this is by no means its only role). Broadly, the action of α_2-receptors is opposite to that of β-adrenoceptors - they are coupled to Gi/o proteins and the Gi protein inhibits the cAMP-PKA signalling pathway. Additionally, activation of the βγ Gi subunit increases the potassium conductance of the

cell and the Go protein supresses voltage-gated calcium channels and depresses the amplitude of the HCN current. Both of these actions have the effect of reducing neurotransmitter release when the α_2-adrenoceptor is presynaptic (Fig. 1.5).

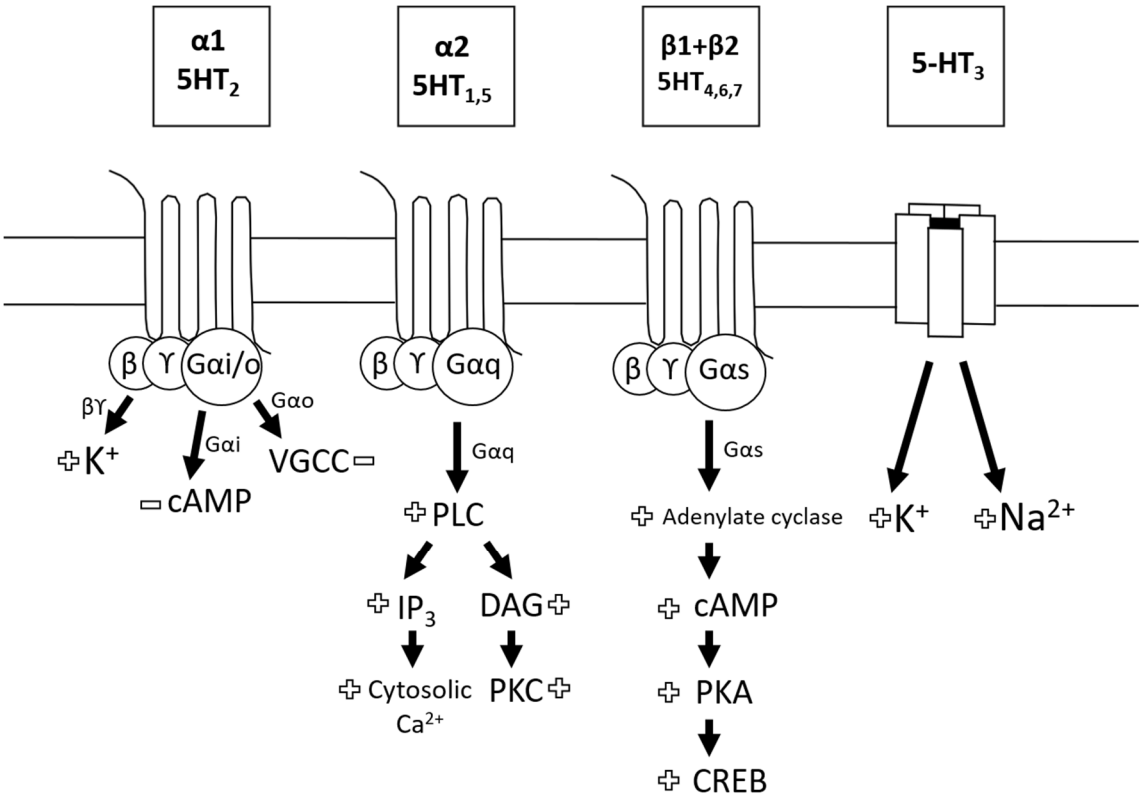


Fig. 1.5: Major intracellular signalling pathways of 5-HT and Noradrenaline receptor subtypes. Major intracellular signalling pathways of the metabotropic G-protein coupled 5-HT and NA receptors. 5-HT_3 receptor is a ligand-gated anion channel.

The three types of adrenoceptor have different affinities for NA, in this order: $\alpha_2 > \alpha_1 >$ β-adrenoceptors. These differences in affinity have functional implications because the magnitude and frequency of NA release can be a determinant of which adrenoceptors are activated in different regions and under different circumstances (see Hein, 2006 and Marzo et al, 2009 for detailed discussions of receptor physiology).

1.3.2 5-HT: General anatomy and receptor pharmacology

In comparison to the relatively restricted origins of noradrenergic afferents there are a large number of serotonergic nuclei scattered widely across the brainstem. As the

main source of noradrenergic afferents, the LC contains only noradrenergic projection neurons. In contrast, most of the 5-HT containing nuclei contain as many or more projection neurons of different neurochemical identity so care is needed in confirming the identity of neurons in the serotonergic nuclei when examining afferent projection systems. Within the brain, the majority of serotonergic neurons are found in the raphe nuclei, a group of nuclei centred on the midline of the brainstem and considered to be reticular nuclei, and additional groups of serotonergic neurons are present in several other reticular nuclei. These reticular nuclei can be divided into two populations based on brainstem location: the rostral group, including nucleus linearis, dorsal raphe, medial raphe, raphe pontis and nucleus centralis oralis and the caudal group, including raphe magnus, raphe pallidus, raphe obscuris, para/gigantocellular nuclei and intermediate reticular nuclei. Like the noradrenergic afferents, serotonergic afferents innervate a large number of regions including the cerebral cortex, thalamus, hypothalamus, amygdala, hippocampus, substantia nigra, olfactory bulb, cerebellum, other brainstem nuclei and spinal cord.

The cerebellar 5-HT afferent system is estimated to be the third largest afferent set, comprising approximately 15% of cerebellar afferent fibres (Kerr and Bishop, 1991). Although the major source of serotonergic afferents in extracerebellar brain regions are the raphe nuclei, this is not the case for the cerebellum. Using retrograde horseradish peroxidase (HRP) tracing, combined with immnohistochemical identification of 5-HT expressing neurons, several studies have shown that the majority of cerebellar cortical 5-HT afferents originate from other reticular nuclei with only a small fraction originating from the raphe nuclei (Bishop and Ho, 1985; Walker et al, 1981 and Kerr and Bishop, 1991). Source neurons for cerebellar cortical serotonergic afferents were identified in: gigantocellularis, paragigantocellularis, lateral, paramedian and oral pontine reticular nuclei and the periolivary reticular formation with only a small number of neurons observed in the raphe magnus and median raphe. Additionally, both Bishop and Ho (1985) and Kerr and Bishop (1991) observed that only a very small percentage of the HRP-labelled cerebellar afferents in the source nuclei (often <5%) co-labelled for 5-HT. The serotonergic afferent input to the cerebellar nuclei appears to be different. Kitzman and Bishop (1994) found that all three cerebellar nuclei contained afferents from the serotonergic raphe nuclei. All three main cerebellar nuclei received afferents from dorsal raphe and dorsal tegmental nucleus; the fastigial nuclei received specific afferents from raphe magnus and peri-olivary reticular formation; interposed from raphe magnus and the lateral nuclei from the median raphe. This differential projection to cortex and nuclei from non-raphe and raphe sources, respectively raises the possibility

that the cerebellar nuclei and cortex process different information signalled by the serotonergic system.

One characteristic feature of serotonergic afferents in other brain structures is a high degree of fibre collateralisation and Jones (1975) suggests that, when this afferent system is traced with HRP, the extent of retrograde labelling is dependent upon the number of afferent terminals for each fibre present within the injection site. If serotonergic afferents branch significantly and project terminals across large areas of the cerebellar cortex, then few sites for HRP uptake will be present for each afferent fibre. This deficiency could lead to an underestimation of the population of serotonergic source neurons by revealing only those that have fewer collateralising axons. Injection of HRP into the cerebellar nuclei covers the white matter where serotonergic afferents fibre bundles are concentrated as they pass onto the cerebellar cortex. Therefore it may be that the nuclei afferents do not represent a distinct population but instead that neurons labelled after nuclear injections are more likely to reflect the entire population of cerebellar serotonergic afferents.

Serotonergic afferents to the cerebellum have been identified in all three layers of the cerebellar cortex and all three cerebellar nuclei (Chan-Palay, 1975; Beaudet and Sotelo, 1981; Takeuchi et al, 1983; Bishop and Ho, 1985; Kerr and Bishop, 1991; Kitzman and Bishop, 1994; Sur et al, 1996). A more comprehensive review of the cerebellar serotonergic afferent system will be found in Chapter 3.

There are seven 5-HT receptor families and 14 subtypes in total. The 5-HT_3 subtype is the only 5-HT receptor that is not a G-protein coupled metabotropic receptor - it is a ligand-gated ion channel. The seven families are 5-HT_1 to 5-HT_7, the subtypes are: 5-$HT_{1A, B, D, E}$ and $_F$; 5-$HT_{2A, B, C}$; 5-HT_3; 5-HT_4; 5-$HT_{5A, B}$; 5-HT_6 and 5-HT_7. Briefly, 5-HT_1 and 5-HT_5 are Gi/o protein coupled and so inhibit cAMP action. 5-HT_2 is Gq protein coupled and stimulates increases in cytosolic calcium and PKC signalling via activation of IP3 and DAG. 5-HT_3 is a ligand-gated sodium and potassium cation channel and thus has a depolarizing effect on the cell. Finally, 5-HT_4, 5-HT_6 and 5-HT_7 are coupled to the Gs protein and thus mobilize the cAMP-dependent intracellular signalling pathways. 5-$HT_{1A, B}$ and $_D$; 5-HT_{5A} and 5-HT_7 are all known to be autoreceptors, whilst 5-HT_{1B} and $_D$, 5-HT_{2A} and $_C$, 5-HT_3 and 5-HT_4 are all known to be heteroceptors located

on terminals of non-serotonergic neurons (Fig. 1.5. See Barnes and Sharp, 1999 for review).

1.3.3 Noradrenaline: Theoretical perspectives

The noradrenergic afferents of the LC and associated nuclei project broadly across almost every region of the brain including the cerebral cortex, cerebellum, many of the subcortical motor, sensory and limbic nuclei and the brainstem nuclei. Activity in the LC is responsive to stressful or arousing stimuli (Singewald and Philipppu, 1998) and its tonic activity varies with the arousal and wakefulness state of the organism (Aston-Jones and Bloom, 1981), suggesting that at least one of its functions is to broadcast information about levels of arousal and awareness. Foote et al (1975) observed that iontophoretic application of NA enhances auditory cortex activity evoked by species-specific calling whilst inhibiting background firing, so as to improve processing of significant stimuli. Woodward et al (1979) suggested this enhancement of evoked inputs represented a general principle of the LC-NA system. Based on these ideas, Berridge and Waterhouse (2003) have suggested that the LC-NA system improves the ability to process relevant stimuli and ignore irrelevant ones in two ways. First, an indirect effect by increasing general vigilance or facilitating the maintenance of persistent focused attention, depending on the attentional demands of the environment. Second, a direct effect by modulating the facility of sensory (thalamic and cortical), associational (PFC) and affective (limbic structures) regions efficiently to process incoming stimuli while filtering out irrelevant input. Similarly, Sara and Bouret (2012) claim that the LC-NA system facilitates the processing of, and responding to, important environmental information and biological imperatives by global reorientation of cortical network processing (also see Bouret and Sara, 2005 and Corbetta et al, 2008).

In addition to these ‘arousal and attention’ models there is a longstanding and well established literature concerning the function of NA signalling in memory consolidation processes (McGaugh, 2004 and Tully and Bolshokov, 2010) and in mediating plasticity mechanisms at the cellular level (Harley, 2004 and Tully and Bolshakov, 2010). These concepts of NA function can be traced back almost 50 years to those of Seymour Kety (1970). Kety claimed that arousing stimuli, including novel stimuli or “stimuli genetically recognized as significant…” may activate the LC leading to the release of NA throughout the brain, including in regions involved in learning such as the hippocampus, amygdala and cerebellum (Kety, 1970). He suggested that activation of

β-adrenoceptors would then induce increased cAMP synthesis in target cells and regulate protein synthesis in a way that changes signalling in the target cell over the long term and could underpin learning and memory. Similarly, Gold and McGaugh (1975) proposed that “an experience establishes a memory state that is transient (soon forgotten) unless the non-specific physiological response to an experience establishes a brain state that promotes memory storage processes.”

The ‘arousal and attention’ theories summarised above have, in several cases, integrated the proposed attentional functions of the LC-NA system with the proposed memory-promoting effect of NA (Berridge and Waterhouse, 2003; Sara, 2015; Sara and Bouret, 2012). For example, Sara and Bouret (2012) suggest that an LC-NA system that reorients attentional and cognitive resources to spontaneous, unexpected salient or threatening stimuli can, by simultaneously mediating memory processes, lead to the organism learning adaptive behavioural responses to those stimuli. This idea is supported by the observation that LC activity mirrors learning processes and may be causally involved in those learning processes. Noradrenergic LC neurons respond with phasic bursting to the presentation of noxious, novel and behaviourally salient stimuli (Foote et al, 1980; Aston-Jones and Bloom, 1981; Vankov et al, 1995) and this response rapidly habituates. However if the stimuli are associated with an appetitive or aversive reinforcer, the phasic response persists through training and fades again once the association is overtrained (Sara and Segal, 1991; Sara et al, 1994). However, at the onset of extinction or reversal learning or on changing the task parameters, the phasic response to the stimuli will re-appear once more (Sara and Segal, 1991; Bouret and Sara, 2004; Bouret and Richmond, 2009). McIntyre et al (2002) showed that hind-paw electrical stimulation presented in a novel environment led to a sustained release of NA in the amygdala. These findings suggest that the LC response to significant stimuli and the persistent response to stimuli paired with reinforcers leads to a significant increase in noradrenergic signalling in LC terminal fields, as suggested by Kety (1970).

Gilbert (1975) proposed an adaptation to the Marr-Albus model that assigned a specific function to the cerebellar NA afferents, in general agreement with the proposal of Kety (1970). Gilbert hypothesized that the noradrenergic signal could act as a gating signal that would initiate a consolidation process, which would convert short-term plasticity induced by coincident CF and MF signalling into a long-term plasticity under behaviourally relevant conditions.

1.3.4 5-HT: Theoretical perspectives

The source of noradrenergic signalling and the expression patterns of adrenoceptor subtypes in the brain is relatively homogenous, suggesting NA may have a restricted set of unifying functions. In contrast, serotonergic nuclei are heterogeneous, dispersed widely through the brainstem, they receive distinct patterns of input and they have different afferent targets. For example, only a small number of serotonergic raphe neurons project to the cerebellum. So it could be that the 5-HT afferent system has a wide range of more specific functions that relate to its different origin nuclei. However, progress in establishing such differences has been slow. The classification of different 5-HT subtypes has been an ongoing process over the last 25 years and so the inadequacies in detailed mapping of receptor type distribution and pharmacological assessment of the physiology of each receptor type has limited the development of coherent theoretical accounts of the function of the 5-HT system. For example, based on clinical observations of the effects of selective serotonin reuptake inhibitors (SSRIs), which potentiate synaptic 5-HT signalling by blocking reuptake and recycling of released 5-HT), in the treatment of mood disorders such as depression or anxiety, it has been suggested that 5-HT may mediate anxiety. However, it has been shown that activation of the dorsal raphe has distinct and opposing effects on anxiety behaviours in animals then the activation of the median raphe (Teissier et al, 2015). Additionally there is a large amount of local specificity in the expression of receptor sub-types. Reflecting this heterogeneity, there are very few comprehensive theoretical accounts of the 5-HT system such as that of Kety (1970) or Sara and Bouret (2012) for noradrenaline. Most accounts focus on specific roles for serotonergic signalling, including learning and memory mediated by specific receptor subtypes in different brain regions (Harvey, 2003; Meneses and Perez-Garcia, 2007; Ogren et al, 2008; Nichols, 2011; Meneses and Lily-Salmeron, 2012; Tellez et al, 2012). For example, a consistent observation in behavioural research is the disruption of learning by application of 5-HT_{1A} agonists and the improvement of learning, or reversal of learning deficits, with the application of 5-HT_{1A} antagonists. On this basis, Ogren et al (2008) suggests that 5-HT_{1A}-mediated signalling, particularly in the hippocampus, acts to prevent plasticity on neurons that are usually the sites of plasticity in acquisition. In this way, serotonergic signalling could act to define the time periods during which plasticity could or could not take place.

1.3.5 Summary Monoamines: Anatomy, physiology and theoretical perspectives

There are significant afferent inputs to the cerebellum from both noradrenergic and serotonergic nuclei. Theoretical accounts of the function of noradrenergic signalling in the brain are comprehensive in their scope and many propose a role for NA in memory processes, including cerebellum dependent learning processes (Gilbert, 1975). Compared to the NA system, a full description of the 5-HT system is relatively recent. Additionally, the heterogeneity of the serotonergic nuclei and the incomplete accounts of the distribution of 5-HT receptors in the brain has led to less comprehensive theoretical accounts of 5-HT function with a tendency to focus on the function of a specific 5-HT receptor in a particular type of behaviour or physiological function. Some analyses propose a role for individual 5-HT receptors in learning processes (e.g. Ogren et al, 2008) but only one account specifically links serotonergic signalling to cerebellum-dependent learning processes (Schweighofer et al 2004). A discussion of this theoretical account in the context of what is known about the monoaminergic systems in the cerebellum can be found in section 1.6 of this chapter.

1.4 Monoamines in the cerebellum: Noradrenaline

1.4.1 Noradrenaline: Receptor distribution

All layers of the cerebellar cortex and the cerebellar nuclei are innervated by noradrenergic afferents and the effects of noradrenergic signalling on its target cells are mediated by the action of a range of adrenoceptors (α_{1A}, α_{1B}, a_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2, β_3). To know which of these receptors are expressed in the cerebellum and in which cells would provide an insight into the potential physiological actions of noradrenergic signalling in the cerebellum and its possible contribution to cerebellar functions. The expression of a range of adrenoceptors subtypes in the cerebellum has been investigated in different species using a range of methodologies. However, there are considerable gaps in our knowledge of these distributions. A detailed examination of the distribution of the α_1-, β_1- and β_2-adrenoceptors by cell type in the cerebellum is presented in Chapter 2, so a comprehensive review of what is already known about adrenoceptor expression in the cerebellum can be found there.

1.4.2 Noradrenaline: In Vitro/ Electrophysiology effects

Another approach to examining the potential actions of noradrenaline in the cerebellum is to record the physiological consequences of manipulating noradrenergic signalling on activity within the cerebellar microcircuit using electrophysiological techniques.

An early *in vitro* electrohysiological study by Crepel et al (1987) found that application of a α-adrenoceptor agonist led to hyperpolarization of PCs. In a later *in vitro* study Hirono and Obata (2006) observed contrasting effects of α_1- and α_2-adrenoceptor activation on MLI excitability and neurotransmission, with α_1-adrenoceptor activation potentiating GABAergic signalling to PCs and α_2-adrenoceptor activation inhibiting GABAergic signalling. Herold et al (2005) and Hirono et al (2008) made similar observations concerning the effect of α_1- and α_2-adrenoceptor activation respectively. α-adrenoceptor appears to be an important influence on PC activity by modulating inhibitory signalling to the PC.

Electrophysiological investigations of the role of β-adrenoceptors in the cerebellum fall into two main categories: *in vivo* recording from anaesthetised adult animals and *in vitro* recording in slices of juvenile or neonate cerebellar tissue. Results have been broadly consistent within approach but different between approaches. In general, *in vivo* recording studies have found that noradrenergic signalling causes an inhibition of PC activity and facilitates the response of PCs to GABAergic signalling (Hoffer et al, 1971, 1973; Freedman et al, 1976, 1977). The facilitated GABA response mechanism in PCs is probably postsynaptically localised (Moises et al, 1979; Yeh and Woodward, 1983), mediated by activation of the β_1-adrenoceptor (Yeh and Woodward, 1983) and cAMP (Cheun and Yeh, 1992). A similar β-adrenoceptor mediated facilitation of the inhibitory effects of exogenously applied GABA has also been reported for cerebellar nuclear neurons (Gould et al 1997).

Evidence from *in vitro* recording studies is mostly consistent with findings *in vivo*. The predominant effect of noradrenergic signalling on cortical activity is a facilitation of inhibitory signalling to the PC. Where these studies differ is in their identification of the site of NA action. *In vitro* studies have suggested that noradrenergic signalling acts not on PCs directly but on inhibitory interneurons to facilitate their activation and thus potentiate inhibition on the PC (Llano and Gerschenfeld, 1993; Mitoma and Konishi,

1999; Saitow et al, 2000a, b). This interneuron facilitation appears to be mediated by activation of the β_2-adrenoceptor (Saitow et al, 2000a, b; Saitow et al, 2005), rather than by the β_1-adrenoceptor as observed in *in vivo* recordings (Yeh and Woodward, 1983). The reason for the different findings with these two approaches is unclear but may relate to multiple factors including anaesthesia in the *in vivo* studies and the recording environment (including media and temperature) in the *in vitro* studies or in the different developmental stages of animals used in each study.

Some *in vitro* studies have demonstrated an involvement of noradrenaline signalling in plasticity mechanisms in the cerebellum. Lippiello et al (2015) observed an interaction between α-adrenoceptor and β-adrenoceptor activation on PF-PC synaptic efficacy and plasticity. β_2-adrenoceptor activation induced a short-term increase in evoked PF EPSCs and application of a specific β_2-adrenoceptor agonist in concert with a PF train stimulus below threshold to induce LTP without the agonist was enough to induce LTP of the PF-PC synapse. However, application of NA itself led to a short-term decrease in evoked PF EPSCs that was mediated by activation of both α-adrenoceptor types. Additionally, Carey and Regehr (2009) found that application of NA decreases the release probability at CF synapses via activation of α_2-adrenoceptors on CF terminals. This decreased release probability led to a reduction in calcium elevation in the PC after CF activation which caused a decrease in the probability of induction of PF-PC LTD by coincident PF train stimulation and CF stimulation following α_2-adrenoceptor activation. If these effects are present *in vivo* they would allow noradrenergic signalling to the cerebellum to modulate plasticity with potential importance for memory acquisition.

Consistent with the theory that noradrenergic signalling is critical to long-term consolidation of memory (Kety, 1970; Gilbert, 1975), hippocampal β-adrenoceptor mediated signalling has been found to be important for the induction and expression of persistent forms of induced plasticity. *In vitro* studies have demonstrated that a form of LTP in the perforant path to dentate gyrus synapse lasting 20-45 minutes is induced by application of NA or a β-adrenoceptor agonist that activates the cAMP-PKA pathway (Harley, 2004). The same LTP can be induced *in vivo* in anaesthetised rats by LC stimulation (Harley and Milway, 1986). However, these short- lived plasticity mechanisms are unlikely to fully capture the critical mechanisms that underpin memory consolidation *in vivo*. In a study that examined plasticity mechanisms over a much longer time period, similar to the time periods that operate in *real* learning and memory

mechanisms, LC stimulation in awake behaving rats led to a short-term (3 hour) and long-term (at least 24 hours) modulation of the perforant path to dentate gyrus synapse. Intraventricular infusions of propranolol or anisomysin before LC stimulation selectively prevented the persistent potentiation leaving the transient potentiation intact (Walling and Harley, 2004). These findings suggest an important involvement of β-adrenoceptor mediated protein synthesis in a long-term plasticity mechanism that is independent of short-term plasticity induction and expression.

1.4.3 Noradrenergic signalling: Effects on non-cerebellum dependent learning and memory

A substantial amount of behavioural research has examined the role of noradrenergic signalling in memory acquisition and consolidation in a number of brain regions using a number of different tasks. This main literature is reviewed here, followed by an analysis of evidence to date concerning the involvement of noradrenergic signalling in cerebellum-dependent learning.

Straube and Frey (2003) found that exploration of a novel environment after tetanic stimulation of perforant path to dentate gyrus synapses can convert potentiation of those synapses from a transient form to a persistent form that lasts at least 24 hours and this conversion requires β-adrenoceptor activation. In a one-trial conditioned taste aversion task, pre-training infusion of propranolol to the insular cortex disrupted performance on a long-term recall trial three days after training (Berman et al, 2000). Gibbs and colleagues (Gibbs and Summers, 2002) have demonstrated critical involvement of noradrenergic signalling in two regions of the chick brain, functionally equivalent to the basal ganglia, in consolidation of a one-trial taste discrimination task. According to Gibbs and Ng (1976), post-training consolidation processes in this task can be split into distinct phases and development of each phase relies differentially on β_2- or β_3-adrenoceptor activation.

In another example of time-dependent consolidation processes, Gallagher et al (1977) found that immediate, post-training, intra-amygdala infusions of propranolol in an inhibitory avoidance task led to a retention deficit that was not present if the infusion was delayed by six hours. Similarly, Liang et al (1986) found that immediate, but not 3 hour delayed, post-training intra-amygdala infusions of NA enhanced memory

retention. The use of single trial learning tasks can reveal a differential role for noradrenergic signalling in long-term and short-term recall, similar to the difference in short- and long-term plasticity observed by Harley and Millway (2004). In a one-trial inhibitory avoidance task, Izquierdo et al (1998) found that post-training microinjections of NA into the CA1 region of the hippocampus had no effect on short-term (1.5 hours) retention but improved long-term (24 hours) retention. In addition, Ji et al (2003) found that post-training intra-CA1 propranolol infusions five minutes but not six hours after contextual fear conditioning impaired long-term (48 hours) but not short-term (1.5 hours) recall.

β-adrenoceptors are coupled to, and activate, the Gs protein which triggers the cAMP-dependent PKA pathway and can lead to activation of transcription factors that regulate gene expression and are thought to be a critical component of memory formation. In addition to evidence for the importance of β-adrenoceptor-mediated processes in memory retention, there is some evidence that this mechanism is at least partly reliant on the cAMP-dependent PKA pathway. For example, in an odour preference task in rat pups that has been shown to be reliant on LC activation, noradrenergic signalling and β-adrenoceptor activation in the olfactory bulb (Sullivan et al, 1989; Yuan et al, 2000; 2003) 24-hour retention of learning depends on cAMP elevation and phosphorylation of CREB in the olfactory bulb (Yuan et al, 2000; 2003)

1.4.4 Noradrenergic signalling: Effects on cerebellum-dependent learning and memory

In addition to the importance of noradrenergic signalling for learning and memory processes in other regions of the brain, there is evidence that implicates noradrenergic signalling in cerebellum-dependent learning tasks. In one of the earliest studies, Watson and McElligot (1983) examined performance of rats in a rod-running task claimed to be cerebellum-dependent. Intracisternal infusion of 6-hydroxydompamine (6-OHDA) that depletes NA and DA signalling in the CNS impaired acquisition of the task over four training sessions, as measured by the time taken to cross the runway on each successive session. This impairment was also seen when NA and DA depletion was restricted to the cerebellum (Watson and McElligot, 1984). Heron et al (1996) confirmed that NA signalling is the critical monoamine in the 6-OHDA disruption. Pre-training systemic application of the β-adrenoceptor antagonist propranolol disrupted acquisition of the task but neither α_1- nor α_2-adrenoceptor antagonists affected the task.

There is also evidence demonstrating a necessary role for β-adrenoceptor-mediated NA signalling in the VOR adaptation and classical conditioning of the NMR/EBR - two learning tasks with independent evidence that they are cerebellum dependent.

Deficits in adaptive VOR gain increase were recorded by McElligott and Freedman (1988) after bilateral intracisternal 6-OHDA injections. But the study was able determine if the effect was mediated by disruption of dopaminergic, or noradrenergic signalling, or both. In a series of experiments, Pompeiano (summarised by Pompeiano, 1998) showed that intravermal infusions of a β-adrenoceptor agonist increased the gain of the vestibulospinal reflex (a reflex which is modulated by PC output from regions of the anterior vermis) whilst infusions of a β-adrenoceptor antagonist had the opposite effect. Similarly, intrafloccular infusions of a β-adrenoceptor agonist and antagonist increased and decreased gain of the VOR, respectively. Pre-training application of the antagonist prior to tasks which induce adaptation of the vestibulospinal reflex or VOR inhibit the adaptation of either reflex, whilst pre-training agonist application amplify the adaptive response.

Winsky and Harvey (1992) conducted one of the first examinations of the impact of disrupted monoaminergic signalling on NMR conditioning. They gave bilateral, intraventricular injections of 6-OHDA and found the highest dose led to a significant disruption in CR acquisition without any significant effect upon the UR or upon the threshold to elicit a CR or upon non-associative responding. Gould (1998) examined the function of β-adrenoceptor activation in NMR/EBR conditioning to an auditory tone CS in rabbits. He found that 5 and 10mg/kg systemic propranolol impedes learning when applied 30 minutes before training, but 1 and 3mg/kg doses had no significant effects. The effective 5mg/kg dose did not affect the UR, topography or timing of the CR or auditory sensitivity. Although the propranolol was systemically applied, the evidence that the cerebellum is essential for NMR/EBR in rabbits suggests a potential function of cerebellar β-adrenoceptor activation in this learning. Cartford et al (2002) used similar systemic pre-training infusions of propranolol to examine the effects on classical conditioning of the EBR in rats and they replicated the dose-dependent disruption of CR acquisition.

Based on previous work identifying lobule HVI and the interpositus nucleus as critical in rabbit NMR/EBR conditioning (e.g. McCormick and Thompson, 1984; Yeo et al, 1985a;

Attwell et al, 2002b; Cooke et al, 2004; Kellett et al, 2010), Cartford et al (2004a) made relatively large infusions of propranolol, targeted to influence the interpositus nuclei and lobule HVI and observed a significant disruption in the acquisition of rat EBR conditioning. Intra-cerebellar, pre-training infusions of a cAMP inhibitor had similar effects on acquisition, suggesting that β-adrenoceptor activation of the Gs protein and initiation of the cAMP-PKA pathway may be important for this learning.

The use of pre-training infusions of β-adrenoceptor agonists does not allow a distinction to be drawn between the involvement of β-adrenoceptor activation in acquisition and the sort of consolidation processes proposed by Gilbert (1975). In a more direct test of this idea, Paredes et al (2009) gave propranolol infusions 5, 60 and 120 minutes post-training in a classical conditioning of EBR task in rats, to test the role of noradrenergic signalling in consolidation. Post-training propranolol at all three time points over six daily training sessions led to disrupted CR acquisition when compared to infusion control animals. Importantly, however, a separate group of rats underwent *in vivo* microdialysis to examine extracellular levels of NA in the cerebellum before, during and after each of the six daily sessions. Cerebellar NA levels began to increase from baseline at the first time point tested, ten minutes after initiation of the training session and remained at above baseline levels for 80-90 minutes, equivalent to about 1-hour post-session. This NA elevation was specific to associative effects as animals that underwent unpaired, pseudoconditioning training had no change in cerebellar extracellular NA levels at any time point during or after the session. This strengthens the possibility that post-training propranolol disruption of learning might occur through disruption of a cortical specific noradrenergic consolidation signal. However, the results of these studies need to be interpreted with caution. The cannula placements and infusion volumes were specifically chosen in order to spread to the cerebellar nuclei regions underlying lobule HVI, additionally the volume of drug infused and strongly lipophilic nature of propranolol make it likely that the drug spread beyond lobule HVI to other regions of the cortex and beyond the AIP to other cerebellar nuclei. This prevents reliable identification of the critical cerebellar compartment. This point is further reinforced by the observation that infusions delayed by 120 minutes also disrupt CR consolidation, even though the authors reported that NA levels in the cerebellar cortex return to pre-training levels well before 120 minutes. Observations by Cooke et al (2004) that muscimol-sensitive cerebellar cortical consolidation processes are complete by two hours are consistent with the duration of NA elevation, but the 120-minute delayed propranolol effects on consolidation (Paredes et al, 2009) are not. They may therefore be a result of spread of the drug to the cerebellar nuclei. Additionally

propranolol has been shown to block neuronal voltage-gated sodium channels so some or all aspects of the observed disruption may be as a result of this non-noradrenergic action.

Kellett and Yeo (2007) examined the role of noradrenergic signalling in consolidation of classical conditioning of the NMR in rabbits. They made post-training infusions of the β-antagonist atenolol targeted to the medial base of Lobule HVI to reversibly disrupt noradrenergic signalling in the eye-blink control region (Mostofi et al, 2010) during consolidation. They observed a strong disruption of consolidation from immediate post-training atenolol infusion on two consecutive training sessions. Once the post-training infusions were terminated the animal learnt as normal and infusions of atenolol did not disrupt the performance of pre-acquired CRs. Atenolol infusions delayed by two hours were ineffective at disrupting consolidation, this is in agreement with the consolidation time window observed by Cooke et al (2004) and the *in vivo* microdialysis data from Paredes et al (2009). This suggests the effect seen by Kellett and Yeo (2007) is a specific disruption of a cortical noradrenergic consolidation signal. Atenolol has no reported non-specific effects on neural signalling pathways, it is a highly hydrophilic drug which limits its mobility after infusion and the infusion volumes used have previously been determined to have minimal spread into the underlying cerebellar nuclei. All of these factors may have contributed to the time-specific disruption of consolidation reported by Kellett and Yeo (2007).

1.4.5 Noradrenaline in the cerebellum: Summary

There are a range of adrenoceptor subtypes which can be activated by NA, many of which are also present in the cerebellum. Electrophysiological studies have consistently demonstrated an effect of noradrenergic signalling on GABAergic signalling in the cerebellar cortex and nuclei, and in the hippocampus electrophysiological studies have demonstrated the importance of noradrenergic signalling to long-term plasticity mechanisms. In behavioural studies, noradrenergic signalling, particularly that mediated by β-adrenoceptor signalling has been shown to be critical in consolidation processes, particularly consolidation of long-term memory. Activation of the cerebellum β-adrenoceptor has been shown to be critical for the consolidation of classical conditioning of NMR.

1.5 Monoamines in the cerebellum: 5-HT

1.5.1 5-HT: Receptor distribution

A wide range of 5-HT receptor types are expressed in the brain and several of these are known to be expressed in the cerebellum. A detailed analysis of the regional and cellular distribution of these receptors by type within the cerebellar microcircuit is important for understanding the roles of 5-HT in cerebellar function.

Early immunohistochemistry (IHC) and in-situ hybridization studies (ISH) provided inconsistent evidence for the expression of 5-HT_{1A} in the cerebellum (Kia et al, 1996; Miquel et al, 1994). A recent IHC study found 5-HT_{1A} immunoreactivity (IR) in the PCs (Rasul et al, 2013) and Boschert et al (1994) observed 5-HT_{1B} expression in PCs. Two studies reported 5-HT_{2A} IR in what appeared to be PC soma and dendrites (Maeshima et al, 1998 and Cornea-Herbert et al, 1999). This finding was confirmed by Geurts et al (2002) who combined IHC for the 5-HT_{2A} receptor with IHC for cell specific marker proteins to unambiguously confirm the presence of 5-HT_{2A} IR in PC soma and dendrites. Immunoreactivity for 5-HT_{2B} is also reportedly restricted to PCs (Choi and Maroteaux, 1996 and Duxon et al, 1997).

Geurts et al (2002) also observed 5-HT_3 IR in the cerebellum. They reported a small number of 5-HT_3 IR varicose fibres in the ML and GCL of the cerebellar cortex and within the cerebellar nuclei. They speculated that these receptors may act as heteroreceptors, expressed on non-serotonergic axons (potentially noradrenergic, dopaminergic or cholinergic) based on data of their action as heteroreceptors that modulate release of non-serotonergic neurotransmitters elsewhere in the brain.

Pasqualetti et al (1999) and Oliver et al (2000) found 5-HT_{5A} expression in the cerebellar cortex and nuclei using ISH and IHC, respectively. Using fluorescence microscopy and double IHC labelling with cell specific markers Geurts et al (2002) demonstrated the presence of 5-HT_{5A} IR in PCs, MLIs, GoCs and in the dentate nucleus. Finally, Gerard et al (1997) reported the presence of 5-HT_6 IR in the GCL and ML and Geurts et al (2002) and Lippiello et al (2016) reported 5-HT_7 IR in the PC

soma. See table 1.1 for a summary of the 5-HT receptor subtypes that have been postitively identified in the cerebellum.

1.5.2 5-HT: In Vitro electrophysiological effects

Armstrong et al (1987) and Maura et al (1986; 1988) observed effects of 5-HT on GC activation. Maura et al (1986; 1988) suggested that this effect may be mediated by one of the 5-HT_2 receptor subtypes. However the findings from the two studies are inconsistent. Armstrong et al (1987) observed a range of effects on GC activity but Maura et al (1986; 1988) consistently saw an inhibition of GC activation. Maura et al (1986; 1988) also observed a consistent reduction of glutamate release at PF terminals following 5-HT_1 activation on PF terminals and Thellung et al (1993) saw an inhibition of MF signalling. Taken as a whole, these studies suggest that 5-HT signalling may act as an inhibitory modulator of the MF to GC to PC pathway. Further, Lee et al (1985) and Hicks et al (1989) showed a 5-HT mediated reduction in the excitatory response of PCs to glutamate.

Receptor Subtype	**Expression (Cell/ Structure/ Region)**	**Study**
5-HT_{1A}	Purkinje cell	Rasul et al, 2013
5-HT_{1B}	Purkinje cell	Boschert et al, 1994
5-HT_{2A}	Purkinje cell	Geurts et al 2002; Maeshima et al, 1998; Cornea-Herbert et al, 1999
5-HT_{2B}	Purkinje cell	Choi and Maroteaux, 1996; Duxon et al, 1997
5-HT_3	Varicose fibres	Geurts et al, 2002
5-HT_{5A}	Purkinje cells, Molecular layer interneurons, Golgi cells and cerebellar nuclei	Pasqualetti et al, 1998; Oliver et al, 2000; Geurts et al, 2002
5-HT_6	Granule cell layer and molecular layer	Gerard et al (1997)
5-HT_7	Purkinje cells	Geurts et al, 2002; Lippiello et al, 2016

Table 1.1: Reported distributions of 5-HT receptor subtypes in the cerebellum.

Application of 5-HT in the cerebellar cortex has also been shown to reduce spontaneous activation of PCs (Darrow et al, 1990; Kerr and Bishop, 1992) and enhance GABAergic transmission from interneurons to PCs (Kerr and Bishop, 1992; Mitoma and Konishi, 1996; 1999). Mitoma and Konishi (1999) were unable to mimic the effects on GABAergic signalling with selective 5-HT_{1A}, 5-HT_2, 5-HT_3 or 5-HT_7 agonists but the reduction in PC activity was shown to be mediated by 5-HT_{1A} or 5-HT_7 receptors (Darrow et al, 1990). When a 5-HT_{1A}/ 5-HT_7 antagonist was co-applied with 5-HT the reduction in PC activity was replaced by an increase in activity, suggesting that there are several functional 5-HT receptor types on the PC (supported by anatomical observations) but 5-HT_{1A} and 5-HT_7 usually dominate. In summary, in most electrophysiological studies to date, the default effect of 5-HT on the cerebellar cortical microcircuit is a net decrease in PC activation through a reduction in glutamatergic signalling in the MF to GC to PF pathway, a reduction of spontaneous PC activity and an enhancement of GABAergic signalling to PCs.

Lippiello et al (2016) examined functions of the 5-HT_7 receptor. They were able to induce PF-PC LTD *in vitro* solely with the application of a 5-HT_7 agonist and show that the same agonist blocked LTP induction by a PF only stimulation protocol. Application of a selective 5-HT_7 antagonist blocked PF to PC LTD induction. The findings suggest that 5-HT acting at the 5-HT_7 receptor can bias PF-PC plasticity towards LTD.

A range of serotonergic signalling effects of on cerebellar nuclear activity has been observed. For example Di Mauro et al (2003) reported either inhibitory, excitatory or biphasic responses to iontophoretically applied 5-HT in the dentate and interpositus nucleus neurons, but a consistent inhibitory effect on neurons in the fastigial nucleus that was mimicked by 5-HT_{1A} and 5-HT_2 activation. Murano et al (2011) saw a decrease in evoked EPSCs in large projection neurons of the cerebellar nuclei after 5-HT application, whilst Saitow et al (2009) saw a 5-HT_{1B} receptor mediated inhibition of GABAergic release onto large projection neurons. Such studies have consistently observed 5-HT mediated effects on nuclear activation but no clear picture of the functional effect of 5-HT in the cerebellar nuclei emerges.

More recently, Oostland et al (2011; 2013; 2014) reported a transient increased expression of 5-HT_3 receptor in the early postnatal period compared to adult levels of expression and a developmental specific pattern of 5-HT_{2A} and 5-HT_{1A} expression. The

combination of these factors creates a pattern of cerebellar activation in response to 5-HT that is specific to the early postnatal period and the authors found evidence that this particular expression pattern and the physiological consequences have a significant impact on cerebellar circuit organisation including the development of PF-PC and CF-PC synapses.

1.5.3 5-HT and the Lugaro cell

The Lugaro cell is a large, fusiform interneuron. Of its two axonal projections one projects transverse to the long axis of the folium and the other projects parallel to the long axis. Based on the length of the long axis parallel-oriented axon, Dieudonné and Dumoulin (2000) suggest that it is likely to span multiple microzones. Lugaro cells are not spontaneously active in rat cerebellar slice and the main excitatory driver for their activation is 5-HT (Dieudonné and Dumoulin, 2000; Dean et al, 2003). Both electrophysiological (Dieudonné, 2001) and anatomical studies have failed to identify the 5-HT receptor subtype expressed by Lugaro cells (Geurts et al, 2002). However, 5-HT activation of Lugaro cells is not blocked by a broad spectrum 5-HT antagonist that has no affinity for 5-HT_6 (Dieudonné, 2001) and a low resolution examination of the rat brain reports 5-HT_6 IR in the GCL and ML (Gerard et al, 1997), suggesting 5-HT_6 is a possible candidate receptor. The connectivity of the Lugaro cell, with inhibitory inputs to PCs, GoCs and MLIs, provides a specific gateway for 5-HT to influence processing in the cortical microcircuit.

1.5.4 Serotonergic signalling: Effects on non-cerebellum dependent learning and memory

As a result of the historical lack of available 5-HT receptor subtype-specific drugs, much of the evidence for the involvement of 5-HT in learning and memory has depended upon examining broad disruptions of serotonergic signalling, including treatments that deplete levels of tryptophan, a 5-HT precursor, across the brain. For example, reduction in dietary tryptophan leads to impaired performance on object recognition memory and contextual fear conditioning tasks but not on performance in spatial memory tasks (Lieben et al, 2004; Uchida et al, 2007). Additionally, Ogren et al (1985) showed that pre-training systemic application of *p*-chloroamphetamine, which induces 5-HT release at low doses, impaired active and passive avoidance learning.

The *p*-chloroamphetamine induced 5-HT release can be blocked by SSRIs and the learning impairments were blocked by SSRI administration.

The effect of 5-HT_{1A} activation on memory processes has been particularly well studied for two reasons. The receptor is notably densely expressed in brain regions including the prefrontal cortex, hippocampus and amygdala that are strongly linked to memory processes and historically it has been the only 5-HT receptor subtype for which a selective ligand, 8-OH-DPAT, has been available (Meneses and Perez-Garcia, 2007 and Ogren et al, 2008). The systemic pre-training application of 8-OH-DPAT disrupts learning in spatial tasks including the radial arm and water maze (Winter and Petti, 1987; Carli and Samamanin, 1992; Carli et al, 1992). 8-OH-DPAT also affects fear conditioning tasks, e.g. 24-hour recall in a place avoidance task is changed in a dose-dependent manner - low doses of 8-OH-DPAT facilitate recall and high doses disrupt recall (Luttgen et al, 2005). In examining local effects that could mediate the disruptive effect of 5-HT signalling on spatial and emotional memory tasks, pre-training intra-amygdala infusions of 8-OH-DPAT have been shown to impair inhibitory avoidance learning (Liang et al, 1999) and intra-hippocampal infusion disrupt learning in a spatial cued avoidance task (Barros et al, 2001), as well as place avoidance (Carli et al, 1993).

Based on the consistent observation that the application of 5-HT_{1A} agonists disrupts acquisition in learning tasks, Ogren et al (2008) suggest that a key function of 5-HT signalling is to delay memory encoding processes. Supporting this view are the observations that application of 5-HT_{1A} antagonists improves learning in several tasks (Schneider et al, 2003) including spatial learning following local intrahippocampal antagonist infusions (Belcheva et al, 1997). Additionally, the application of a 5-HT_{1A} antagonist can reverse learning deficits induced by disruption of other neurotransmitter systems, including disruption of spatial learning by blockade of hippocampal NMDA receptors (Carli et al, 1999) or muscarinic receptors (Carli et al, 1996; Pitsikas et al, 2003; Egashira et al, 2006). Again, this evidence suggests that 5-HT may normally function as an inhibitory signal, delaying plasticity processes so that, here, the release of this signal allows the remaining plasticity processes available to the cell to overcome the loss of muscarinic and NMDA receptor activation. Finally, in addition to these analyses of acquisition processes, one study has indicated a potential role for 5-HT_{1A} activation in consolidation. Prado-Alcala et al (2003) found post-training infusions of 5-HT in the striatum produced amnesia for a passive avoidance task.

There has been some behavioural research using a range of agonists and antagonists for other 5-HT receptor subtypes, including relatively selective 5-HT_4, 5-HT_6 and 5-HT_7 selective ligands (King et al, 2008; Roberts and Hedlund, 2012). Similarly to 5-HT_{1A}, 5-HT_6 activation appears to be disruptive to acquisition processes. Rogers and Hagan (2001) found pre-training infusions of selective 5-HT_6 antagonists significantly increased seven-day retention in a water maze task. However, the findings in this literature are often contradictory or unclear. For example Woods et al (2012) saw no facilitatory or inhibitory effects of application of a 5-HT_6 agonist or antagonist on a conditioned fear task but systemic post-training application of either an agonist or antagonist reversed the memory deficits induced by pre-training infusions of muscarinic or NMDA antagonists. So it is unclear whether the effects seen following systemic application of 5-HT_6 ligands are as a result of a specific effect on 5-HT_6 activation or the result of a non-specific drug effect. This level of inconsistency is likely to be due in part to the use of a wide range of drugs, dosages and learning paradigms. Additionally, there is an over-reliance on systemic pre-training drug injections or, more recently, in the use of transgenic 5-HT receptor knockout mice as a means of examining the involvement of 5-HT receptor activation in learning and memory processes. Using these approaches, it is not possible to confirm whether any disruption in memory task performance is due to disruption of a specific 5-HT mediated associative process or a general disruption to circuit function (Roberts and Hedlund, 2012). This is particularly critical in the examination of the effects of 5-HT_{1A}, because 5-HT_{1A} is an autoreceptor that is expressed somatodendritically on serotonergic raphe neurons (Barnes and Sharp, 1999). Even if the behaviour under investigation has been previously demonstrated to be highly reliant on a particular brain region, the use of systemic 5-HT_{1A} ligand infusions make it impossible to distinguish whether the effects are upon 5-HT_{1A} activation in the regions of interest or whether they are regulatory at the source nuclei.

The effects of systemic and local infusions of SSRIs have been studied in several forms of one-trial avoidance learning tasks. In inhibitory and active avoidance tasks, systemic SSRI infusions given before retention tests have been shown to improve recall and post-training infusions enhance long-term retention in both tasks (Altman et al, 1984; Flood and Cherkin, 1987). In an inhibitory avoidance task, recall was enhanced by post-training systemic infusions of the SSRI fluoxetine but not by intra-amygdala infusions of the same drug (Introini-Collison et al, 1992). However post-training intra-septal infusions did improve recall, suggesting serotonergic signalling in the septum is important for inhibitory avoidance learning (Lee et al, 1992). Additionally,

post-training systemic infusion of fluoxetine has been shown to improve retention in an autoshaping task (Meneses and Hong, 1995).

1.5.5 Serotonergic signalling: Effects on cerebellum-dependent behaviours

Several studies have indirectly linked serotonergic signalling to cerebellar processes. For example, in clinical studies of patients with cerebellar ataxias, Trouillas et al (1988) found chronic administration of the 5-HT precursor L-5-hydroxytryptopha (5-HTP) improved patient ataxia scores and performance in other movement coordination tests. In Lurcher mutant mice, long-term treatment with 5-HTP or a 5-HT_{1A} agonist also led to improvements in motor coordination (Le Marec et al, 2001). Additionally, Veasey et al (1995) recorded from serotonergic neurons in the raphe nuclei obscurus and raphe pallidus and found that a large number of cells responded with increased activity during the motor tasks of treadmill walking and feeding, suggesting that serotonergic signalling may modulate processing in target fields during movement. More specifically for the cerebellum, Mendlin et al (1996) used *in vivo* microdialysis in freely moving rats to reveal that 5-HT release in the cerebellum is modulated by behavioural state. There is a strong positive correlation between active waking states in the rat and cerebellar 5-HT levels, feeding also elevates levels and feeding in the presence of tail pinch elevated 5-HT further. This suggests that 5-HT could modulate cerebellar circuit activity during sensorimotor processes.

Studies using NMR conditioning have provided some insights into 5-HT function in memory. Harvey et al (1988) showed that systemic infusion of different broad spectrum 5-HT agonists enhanced delay NMR conditioning to short ISI (100 ms) and long ISI (800 ms) but not at optimal 200 and 400 ms ISIs. These improvements were due to associative components as several broad spectrum 5-HT agonists led to an improvement in CR acquisition with no effect on pseudoconditioning or baseline responding (Romano and Harvey, 1994). In another study, systemic infusion of a broad spectrum 5-HT agonist had a facilitatory effect on trace conditioning with long trace periods (>1000 ms. Siegal and Freedman, 1988). Finally, systemic infusion of highly specific 5-HT_{2A} or 5-$HT_{2A/C}$ antagonists, but not non-selective 5-HT antagonists disrupted CR acquisition (Welsh et al, 1998 and Romano et al, 2000) suggesting the effect was mediated specifically by 5-HT_{2A} receptors.

Burhans et al (2013) found that five consecutive days of systemic fluoxetine administered after training improved CR retention in delay classical conditioning of the NMR one day and one week after the conclusion of training. Indicating an important role for 5-HT signalling in the consolidation and long-term storage of learning-related changes. In contrast however Leuner et al (2004) found that chronic systemic fluoxetine infusions had no effect on the acquisition of trace eye-blink conditioning in rats.

Thus, the behavioural work examining the function of serotonergic signalling in learning and memory has yielded some inconsistencies. Work using 5-HT_{1A} agonists suggests that signalling via this receptor may have a negative gating effect on acquisition processes. In contrast, other research has shown a positive action of 5-HT_{2A} activation on acquisition of NMR conditioning and work with SSRIs has consistently demonstrated a facilitation of learning in a range of tasks.

1.5.6 5-HT in the cerebellum: Summary

The expression of a range of 5-HT receptor subtypes in the cerebellum has been demonstrated. In particular, there are a number of subtypes expressed by the PC, suggesting that its activity is importantly modulated by serotonergic signalling. Observations from electrophysiological studies appear to support this view and the net effect of serotonin signalling in the cerebellar cortex, measured electrophysiologically, is a decrease in PC activation through an inhibition of spontaneous activity, potentiation of GABAergic signalling and inhibition of glutamatergic afferent signalling. The net result of these effects would be a decrease in PC output to the cerebellar nuclei, potentially disinhibiting cerebellar nuclei activity and so increasing cerebellar output. The data available on the role of 5-HT signalling in learning and memory processes appear to have some inconsistencies. These may be partly due to the use of systemic drug infusions and non-specific ligands in much of the existing research. Better targeted infusions of ligands selective for 5-HT subtypes, similar to the approach used in analysis of noradrenergic signalling, will be an important step in defining the role of serotonergic signalling in learning and memory processes in regions including the cerebellar cortex and nuclei.

1.6 Monoamines in the cerebellum: Open questions

The results of the anatomical and electrophysiological research reviewed here provide evidence that noradrenergic and serotonergic signalling form critical components of normal cerebellar processing and so are important in the execution of cerebellum-dependent behaviours. But few accounts of cerebellar function, whether historical or contemporary, provide a comprehensive analysis of NA and 5-HT function within its well-defined architecture (Marr, 1969; Albus, 1971; Ito, 2006). On the basis of evidence that monoaminergic inputs make a meaningful contribution to cerebellar processing, Schweighofer et al (2004) has proposed one of the few specific models that attempts to account for the function of each of the monoaminergic afferents (5-HT, dopamine, histamine and noradrenaline plus acetycholine) in the cerebellum. It is suggested that 5-HT provides a 'responsibility' signal, coordinating the output of functionally connected microzones by simultaneously modulating their activity. Schweighofer et al (2004) propose that NA afferents in the molecular layer provide a signal to all microzones within the cerebellar cortex simultaneously. This signal is proposed to gate/ control the induction of various forms of plasticity throughout the cerebellum including LTD of the PF-PC synapse.

For noradrenergic signalling, theoretical accounts (e.g. Kety, 1970; Gilbert, 1975 and Schweighofer et al, 2004) and behavioural data are in reasonable agreement. There is a clear and critical role for NA signalling in memory acquisition and consolidation in many regions of the brain, including the hippocampus (Harley and Walling, 2004), amygdala (Liang et al, 1986) and in cerebellum-dependent learning processes (Cartford et al, 2004a; Kellett and Yeo, 2007; Paredes et al, 2009). This evidence allows several important questions to be asked. What physiological mechanisms underpin the noradrenergic consolidation signal within the cerebellar cortex? What adrenoceptor subtypes are present in the cerebellum and activated by the noradrenergic consolidation signal? Are there distinct roles for the different adrenoceptor subtypes in cerebellum-dependent learning? How is noradrenergic signalling distributed across the different regions of the cerebellum? Some of these questions will be answered in this thesis.

In comparison to the noradrenaline system, a coherent and consistent account of potential memory functions of the 5-HT system is lacking. Though Ogren et al (2008) suggested a role for 5-HT in the hippocampus as a signal to curb memory encoding

under specific circumstances, the consistent findings that SSRI application (which would potentiate serotonergic signalling) enhances memory and the findings that 5-HT_{2A} activation may improve memory acquisition in NMR conditioning (Harvey, 2003) suggest a more complex and heterogeneous role for 5-HT signals. The fundamental functional organisation of the cerebellum is well defined, so examination of the 5-HT system within this organisation will be an important first step in defining its potential function. The question addressed here is where and how does the serotonergic afferent system distribute in the cerebellum?

1.7 Aims and outlines of study

This thesis aims to characterise functional aspects of monoaminergic signalling in the cerebellum with an anatomical approach (Chapters 2 and 3) and examine the potential function of noradrenergic signalling in consolidation of cerebellum-dependent learning (Chapter 4). These aims will be achieved by the following experiments:

1.7.1 Examination of the distribution of adrenoceptors in the cerebellum

Adrenoceptors are the critical mediators of noradrenergic signalling but earlier studies that have examined the distribution of adrenoceptors in the cerebellum have used methods that have precluded the unambiguous localisation of adrenoceptors to specific cell-types within the cerebellar microcircuit. Chapter 2 will present data from a study in which immunohistochemistry was used to detect expression of three adrenoceptor subtypes (α_1, β_1 and β_2) in specific cell types within the cerebellum, using additional immunohistochemistry to define the different cerebellar neurons.

1.7.2 Examination of the distribution of monoaminergic fibres in the cerebellar cortex

The distributions of the mossy fibre and climbing fibre afferents in the cerebellar cortex are important determinants of their function and the main functional principle of the cerebellum is defined by the patterned distribution of climbing fibres. Very little is known about the distribution of monoaminergic afferents, in particular in relation to the functional zones of the cerebellum. In Chapter 3, evidence is presented from a study of immunohistochemically identified noradrenergic and serotonergic afferent fibres to reveal their detailed distribution within the cerebellar functional architecture.

1.7.3 Examination of β-adrenoceptor function during consolidation of classical conditioning of the nictitating membrane response

Earlier experimental data has demonstrated that activation of the β-adrenoceptor in the post-training period is critical to the consolidation of classical conditioning of the nictitating membrane response. Two of the three β-adrenoceptors (β_1 and β_2) are

expressed in the cerebellum. In Chapter 4 data are presented from a study examining the specific role of the β_1-adrenoceptor in consolidation of NMR conditioning.

Chapter 2: Distribution of adrenoceptors in the cerebellum

2.1 Introduction

2.1.1 Noradrenergic neurotransmission in the cerebellum and memory consolidation

Gilbert (1975) proposed a specific role for NA as a consolidation signal in cerebellar learning. A body of experimental work has provided evidence for this proposal using pharmacological manipulations of NA signalling to disrupt acquisition of cerebellum-dependent learning processes such as EBR conditioning and VOR adaptation (reviewed in Chapter 1 section 1.4.3). Using targeted pharmacological manipulations, two recent studies have identified β-adrenoceptors in the cerebellar cortex as key mediators of this proposed consolidation signal (Paredes, 2009 and Kellett and Yeo, 2007). The circuitry of the cerebellum has been well defined (particularly for the cerebellar cortex, (Eccles, 1967; Ito, 1984)) and theoretical accounts make specific predictions about the role of the individual cellular components of the circuit for cerebellar learning processes (Marr, 1969; Albus, 1971; Ito, 1972; Gilbert, 1974; Hesslow and Yeo, 2002). If we now wish to investigate how NA might act to consolidate memory formation within these circuits, then a clear description of the distribution of expression of adrenoceptors by the cells of the circuit is essential.

The first aim of the study to be described here is to determine the expression of β_1- and β_2- adrenoceptors by cell type within the cerebellar cortical circuitry. With this information, we may interpret studies that have examined potential β-adrenoceptor mediated cerebellar memory functions that are disrupted by post-training infusions of β-adrenoceptor antagonists (Paredes et al, 2009; Kellett and Yeo, 2007).

A second aim is to examine the expression of the β_1- and β_2-adrenoceptors in the cerebellar nuclei. Very little is known about the distribution of β-adrenoceptors in the cerebellar nuclei though they are known to receive noradrenergic afferents (Hökfelt and Fuxe, 1969; Landis et al 1975). The expression of β-adrenoceptors in the cerebellar nuclei is a potential confounding factor for studies of NA functions in cerebellar cortex using infused antagonists. For example, Paredes et al (2009) blocked consolidation of EBR conditioning with infusions of β-adrenoceptor antagonists at 120 minutes post-training, contrary to their observation that training related NA signalling is completed by 80-90 minutes post-training. This effect may have resulted from spread of the antagonist to the underlying cerebellar nuclei (see Chapter 1, section 1.4.4). As a first step in examining a potential role for nuclear β-adrenoceptors in consolidation, a clear description of their distribution is needed.

Finally, though there has been very little behavioural research specifically examining a role for α-adrenoceptor function in memory processes including consolidation, several *in vitro* studies have identified α-adrenoceptor mechanisms in the cerebellar cortex and nuclei that might be relevant (reviewed in Chapter 1 section 1.4.2). Therefore, an accurate description of the α-adrenoceptor expression is also important for a comprehensive understanding of NA signalling mechanisms in the cerebellar cortical circuit. Thus, the third aim for this study was to use IHC to expand and refine what is currently known about the distribution of α-adrenoceptors in the cerebellum.

2.1.2 The cell type distribution of cerebellar β-adrenoceptors is not fully described

A series of early anatomical studies detected the presence of β-adrenoceptors within the cerebellum largely through the use of three methodologies: ligand binding (usually with radiolabelled ligands), *in situ* hybridization (ISH) and immunohistochemistry (IHC). Using a fluorescent analogue of a β-adrenoceptor ligand, Melamed et al (1976) and later Atlas et al (1977) and also Atlas and Melamed (1978) noted the presence of β-adrenoceptor binding in the cerebellar cortex, though contemporary accounts expressed doubts over the selectivity of the ligand used (Sutin and Minneman, 1985). In several later studies, non-selective β-adrenoceptor radiolabelled ligands in

conjunction with competitive and highly selective β_1 or β_2 agonists were applied to define sub-type specific binding patterns. These studies noted radioligand binding in the cerebellum to both β_1- and β_2-adrenoceptors but the number of β_2-adrenoceptor binding sites was far greater, prompting the conclusion that the β_2-adrenoceptor was the major receptor subtype in rat and guinea pig cerebellum (Palacios and Kuhar, 1982; Rainbow et al, 1984; Johnson et al, 1987; Booze et al, 1989). The results of an ISH study by Nicholas et al (1993) were consistent with this claim: the authors observed β_2 mRNA in the GCL and PCL but no β_1 mRNA in the cerebellar cortex, although they did observe β_1 mRNA labelling in the lateral cerebellar nuclei.

In a very early IHC study Strader et al (1984) reported no β_2 IR in the GCL, PCL or deeper two thirds of the ML but there was moderate β_2 IR in processes in the superficial third of the ML, consistent with the suggestion that there are β_2 receptors on PC dendrites. Wanaka et al (1989) used an antibody reactive with both β_1- and β_2-adrenoceptors. They observed high levels of IR in PCL soma, which may have been PC expression, and in thin processes within the ML as well as scattered IR across the GCL. A more recent whole rat brain IHC study revealed that cerebellar PCL and ML β_1 expression is denser than in any other brain location in contrast to earlier ligand binding studies that consistently reported low levels of β_1 in the cerebellum (Paschalis et al, 2009)

In summary, previous studies have reported both β_1 and β_2 receptor expression in the cerebellar cortex and one study has revealed β_1 expression in the cerebellar nuclei (Nicholas et al, 1993). However these early radioligand and ISH studies lacked the required resolution to report the distributions with precision better than a simple laminar description. Chromogenic or fluorescent IHC does allow for visualisation of receptor expression at resolution sufficient to determine cell specific expression. However only combined fluorescence immunohistochemical detection of a specific receptor and a cell-specific marker protein can unambiguously define receptor expression by cell type, an approach not previously attempted with IHC examinations of β-adrenoceptors in the cerebellum (Strader et al, 1984; Wanaka et al, 1989 and Paschalis et al, 2009).

2.1.3 What is known of the distribution of cerebellar α-adrenoceptors?

α_1-adrenoceptors:

Early radioligand binding studies reported α_{1B}-adrenoceptor but not α_{1A}-adrenoceptor binding in the cerebellar cortex (Palacios et al, 1987 and Wilson and Minneman, 1989). In contrast, *in-situ* hybridization studies have consistently reported the presence of both α_{1B} and α_{1A} mRNA in the cerebellar cortex (Pieribone et al, 1994; Day et al, 1997). Pieribone et al (1994) failed to find α_{1D} in rat cerebellum but Day et al (1997) and Schambra et al (2005) did in rat and human cerebellum, respectively. Schambra et al (2005) also reported all three α_1 subtypes in the dentate nucleus in human. Critically however this ISH methodology lacked the resolution to be determine α_1-adrenoceptor expression by cell type.

Using IHC, Acosta-Martinez et al (1999) reported moderate α_{1B} IR in the PCL, AIP and the dorsolateral protuberance of the medial cerebellar nucleus and high levels in the medial cerebellar nucleus. Papay et al (2004) and Papay et al (2006) used EGFP transgenic mouse lines to reveal α_{1B} and α_{1A} expression in all three cortical layers and α_{1B} expression in the cerebellar nuclei. Thus α_{1A} and α_{1B} expression has been consistently observed in all cerebellar cortical layers in mouse, rat and human. Although α_{1D} in the cerebellum has been less consistently demonstrated, this may reflect the relative insensitivity of *in-situ* hybridization with radio labelling (Pieribone et al, 1994; Day et al, 1997 and Schambra et al, 2005). Until cerebellar α_{1D} is examined with higher sensitivity IHC methods, it is unclear whether its expression is always at the low levels indicated to date.

α_2-adrenoceptors:

In-situ hybridization studies of α_2-adrenoceptors in rat cerebellar cortex consistently report the presence of α_{2A} and α_{2C} but not α_{2B} in the cerebellar cortex (Nicholas et al, 1993; Scheinin et al, 1994; Tavares et al, 1996). Schambra et al (2005) on the other hand reported all three subtypes in the human cerebellar cortex. There are many more IHC studies of α_2-adrenoceptor expression than for α_1- and β-adrenoceptors. In rat

cerebellar cortex, Talley et al (1996) and Aoki et al (1994) have demonstrated α_{2A} IR in soma in the ML (most likely MLIs) and in GCL and PCs in rat and monkey. Also in rat Rosin et al (1996) observed α_{2C} IR in the GCL and ML and small soma in the PCL which are potentially BGC soma based on their size and location. α_{2B} expression was seen in PCs in EGFP transgenic mice (Wang et al 2002). Hirono et al (2008) reported α_{2A} and α_{2B} but not α_{2C} IR in MLIs, PCs and throughout the GCL, this is in contrast to reports in rats of the presence of α_{2A} and α_{2C}, but not α_{2B} in the cerebellar cortex (Nicholas et al, 1993; Scheinin et al, 1994; Rosin et al, 1996; Taveres et al, 1996).

In summary, α_{2A} and α_{2B} appear to be expressed by PCs and MLIs and in the GCL in mice but this pattern of cell expression is seen only for α_{2A} in rats. There may also be a species difference in α_{2C} expression, since α_{2C} is expressed by BGCs and in the ML and GCL in rats (Rosin et al, 1996) but is not expressed at all in mice (Hirono et al, 2008). Thus, the expression of α_2-adrenoceptor subtypes in the cerebellar cortex has been examined in several reliable IHC studies which have been able to localise the expression of different α_2-adrenoceptor subtypes to PCs and MLIs in addition to the GCL (Aoki et al, 1994; Roisin et al, 1996; Talley et al, 1996; Wang et al, 2002; Hirono et al, 2008) whilst the cell specific expression of the α_1-adrenoceptor subtypes is yet to be reliably determined. Importantly, the majority of *in vitro* electrophysiology studies that have reported α-adrenoceptor mechanisms in the cerebellar cortex have found evidence that they are mediated by α_1-adrenoceptors (Crepel et al, 1987; Herold et al, 2005; Hirono and Obata, 2006). Therefore we focused here on describing cell specific α_1-adrenoceptor expression.

2.1.4 Experimental summary

Here, we combined specific β_1- and β_2-adrenoceptor immunohistochemistry with cell marker protein immunohistochemistry to differentiate the distribution of the β_1- and β_2-adrenoceptors in cortical cell types and make a detailed survey of β-adrenoceptor expression in the cerebellar nuclei. We used an antibody raised to a peptide sequence shared by all three α_1-adrenoceptor subtypes to examine the cell type specific distribution of the α_1-adrenoceptor in the cerebellar cortex.

2.2 Methods

2.2.1 Animals and material preparation

Eighteen adult female Sprague Dawley rats (weight: approximately 250 g) were used. All procedures were approved by the local ethical review panel of University College London and were in accordance with the UK Home Office Animals (Scientific Procedures) Act under the provision of licence 70/23405. Each rat was anaesthetised with isoflurane by inhalation (4% in O_2) then killed with an overdose of Euthatal (200 mg/ml pentobarbitone sodium. 200 mg/kg i.p. Merial, Essex). After thoracotomy, each rat was perfused transcardially with approximately 500 ml 0.9% saline containing heparin (500 IU) followed by approximately 500 ml 4% w/v paraformaldehyde (PFA) and 0.5% w/v picric acid in 0.1 M phosphate buffer (pH 7.5). The brains were removed and post-fixed overnight in 4% PFA solution. They were stored for ~72 hours in 20% sucrose/ PFA solution for cryoprotection. Brains were blocked with a coronal cut just rostral to the superior colliculi and the cerebellar block was freeze-mounted onto the stage of a sledge microtome (Leitz 1400; Leica, Germany) and cut in serial 40 µm coronal sections. Sections were rinsed in 0.1 M PBS to remove residual perfusate and stored for no more than 48 hours in 0.1 M PBS at 4°C before immunohistochemical procedures began.

2.2.2 Immunohistochemistry

A blocking solution, of normal goat serum (2.5% w/v), Triton-X100 (0.01% w/v), Tween 20 (0.04% w/v) and bovine serum albumin (1% w/v) in 0.1 M TBS was used in some of the steps below. A blocking solution is a solution that contains a number of reagents that will bind to reactive sites on the tissue section (in this blocking solution bovine serum albumin and normal goat serum), thus reducing the number of available 'non-specific' sites to which the primary or secondary antibody can bind and so reducing the amount of non-antigenic IR. All antibodies were diluted in this blocking solution at the concentrations shown in Table 3.1. All sections were incubated in blocking solution for one hour before incubation in primary antibodies for ~20 hours with agitation (6 hours at room temperature (RT) plus ~14 hours at 4°C). Sections were washed for three, 10 minute incubations in 0.1 M TBS. Sections were then incubated in the appropriate secondary antibodies for two hours at RT. Receptor antibody binding was detected using a goat anti-rabbit biotinylated secondary antibody (β-adrenoceptors) or goat anti-

rabbit Alexa-Fluor 594 secondary antibody (α_1-adrenoceptors). Cell-specific marker antibodies were detected using a goat anti-mouse Alexa-Fluor 488 or Alexa-Fluor 594 secondary antibody. Sections were washed then incubated in Alexa-Fluor 594 (β_1-adrenoceptor) or 488 (β_2-adrenoceptor) conjugated streptavidin diluted in 0.1 M TBS for one hour at RT then washed. Sections were mounted onto microscope slides in VECTASHIELD mounting medium (Vector laboratories, UK) and coverslipped. Coverslips were sealed to the slides and these were stored in aluminium foil to prevent light entry and at 4°C to preserve tissue and fluorescence quality. Sections were imaged with fluorescence or confocal microscopy within seven days in most cases and within two weeks in all cases. Primary omission control sections were prepared using an identical procedure to that described but no primary antibody was applied at any stage (see Fig 2.1). All three receptor antibodies were pre-absorbed with the corresponding control peptide and no non-specific IR was observed (see Fig 2.2).

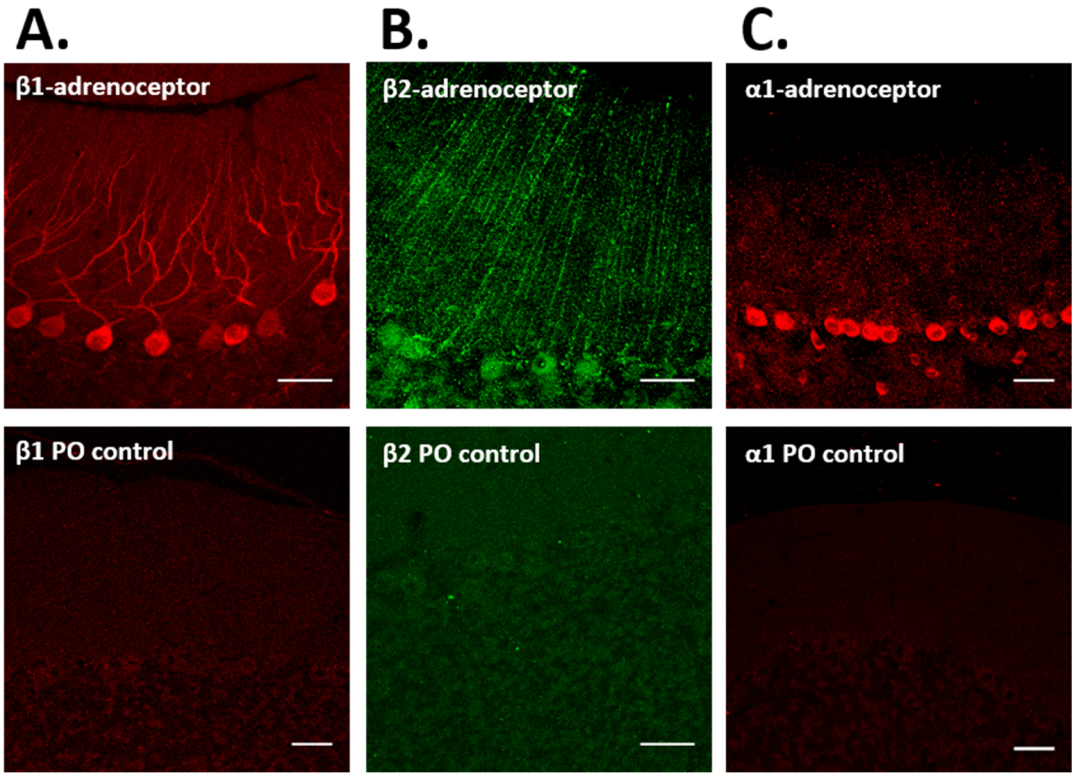


Fig. 2.1: Adrenoceptor primary omission (PO) control sections. (A) β_1-, (B) β_2- and (C) α_1- adrenoceptor PO control experiments revealed very low levels of signal confirming that the patterns of IR observed with primary antibody are specific to the primary antibody. Scale bars: 50μm.

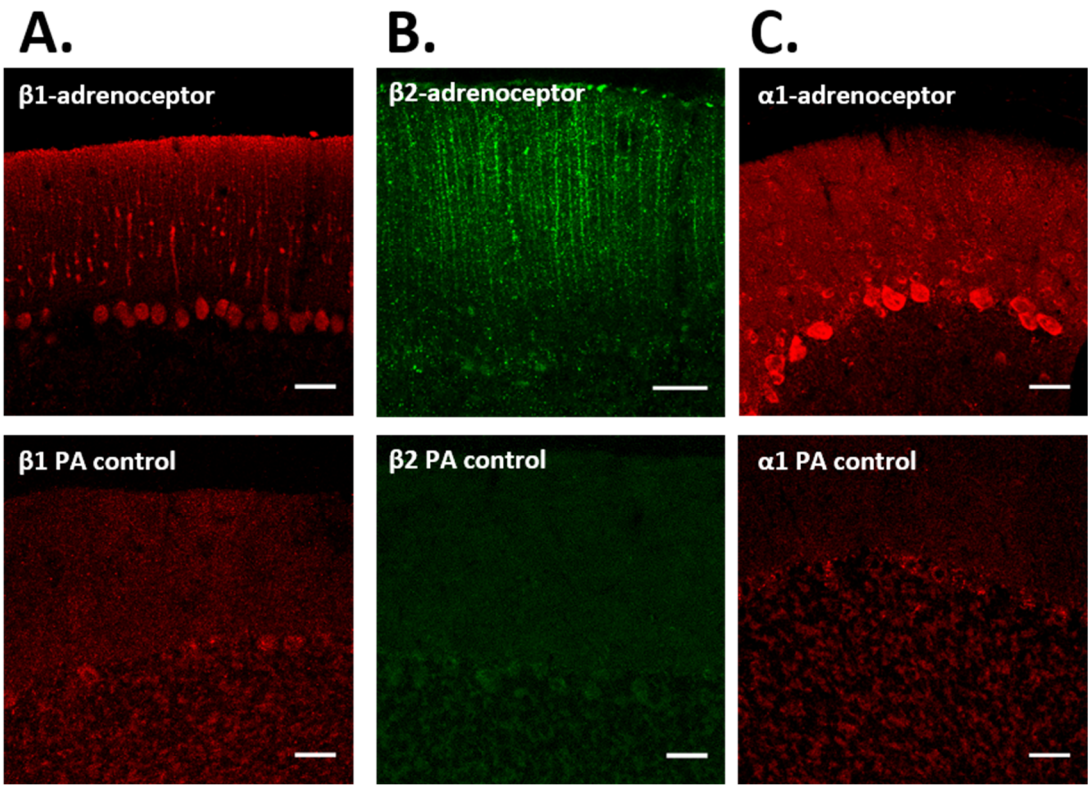


Fig. 2.2: Adrenoceptor peptide absorption (PA) control sections. (A) β_1-, (B) β_2- and (C) α_1- adrenoceptor PA control sections do not show the same pattern of IR as comparable sections without PA, confirming that the IR patterns seen with are due to specifc primary antibody binding. Scale bars: 50μm.

Antigen	Cells labelled	Ab. type	Dilution tested	Final dilution	Secondary/ tertiary detection	Company/ Catalogue #
β_1-adrenoceptor (B1)		Rabbit poly.	1:250; 500; 1000	1:500	Goat biotinylated anti-mouse; AF 594 conjugated streptavidin	SCBT/ sc-568
β_2-adrenoceptor (B2)		Rabbit poly.	1:250; 500; 1000	1:500	Goat biotinylated anti-mouse; AF 488 conjugated streptavidin	SCBT/ sc-569
α_1-adrenoceptor (A1)		Rabbit poly.	1:100; 250; 500	1:250	Goat anti-rabbit AF 594	
Calbindin (CALB1)	PC soma/ dendrites	Mouse mono.	1:500; 1000; 1500	1:1500	Goat anti-mouse AF 488 or 594	Millipore/ AB1778
Zebrin II (ALDOC)	PC soma/ dendrites (Subset)	Mouse mono.	1:500; 1000; 1500	1:1000	Goat anti-mouse AF 488	Gift. Professor Richard Hawkes, Calgary University
Parvalbumin (PVALB)	MLI soma/ dendrites	Mouse mono.	1:500; 1000; 1500	1:1000	Goat anti-mouse AF 488	Sigma-Aldrich/ P3088
β-tubulin III (TUBB3)	General neural marker	Mouse mono.	1:500; 1000; 1500	1:1000	Goat anti-mouse AF 488	Sigma-Aldrich/ T8578
Glial fibrillary acidic *protein (GFAP)*	General glial marker.	Mouse mono.	1:500; 1000; 2000	1:2000	Goat anti-mouse AF 488	Sigma-Aldrich/ G3893
***S100 calcium binding protein β (S100B*)**	BGC soma and proximal dendrites	Mouse mono.	1:1000; 1500; 2000	1:2000	Goat anti-mouse AF 488	Sigma-Aldrich/ S2532
Metabotropic glutamate receptor 2 ($mGlu_2$)	GoC soma and proximal dendrites	Mouse mono.	1:100; 250; 500; 1000	1:500	Goat anti-mouse AF 488	Millipore/ MAB397
Calretinin (CALRT)	LGC, UBC and GC weakly	Mouse mono.	1:1000; 1500; 2000	1:2000	Goat anti-mouse AF 488	Swant/ CR7697
Goat anti-rabbit biotin-XX		Goat poly.		1:200		Invitrogen/ B2770
Goat anti-mouse alexa-fluor 488		Goat poly.		1:400		Invitrogen/ A11008
Alexa-fluor 488 streptavidin				1:1000		Invitrogen/ S11223
Alexa-fluor 594 streptavidin				1:1000		Invitrogen/ S11227

Table 2.1. Summary of primary antibodies and secondary antibody used for their detection. Ab: antibody, mono: monoclonal, poly: polyclonal, AF: Alexa-Fluor.

2.2.3 Fluorescence Microscopy

All initial observations of the labelled sections were made with conventional, fluorescence microscopy using a Zeiss Axioscope with filters optimised to view FITC and Rhodamine fluorophores. Target regions for confocal microscopical examination were identified.

2.2.4 Confocal Microscopy

Digital micrographs were collected using an Olympus FV-1000 TIRF confocal laser scanning microscope with appropriate excitation lasers (488nm and 559nm for the Alexa-Fluor 488 and 594 fluorochromes, respectively). Sections were imaged by collecting successive confocal images at 0.8 µm intervals in the z-plane using a x10 or x20 objective. Images of the primary omission control sections were collected using the same parameters as for the experimental sections with which they are paired in Fig 2.1. The tracking stage of the Olympus microscope allowed high resolution photomicrographs encompassing large areas of the cerebellar nuclei. Coordinates that traced the edges the cerebellar nuclei, or selected nuclear compartments, were specified using the Olympus device software. Based on these coordinates, multiple z-stack images defined by the coordinates were collected and automatically combined by the device software to generate high resolution montage images of the cerebellar nuclei.

All confocal digital micrographs were analysed and prepared for presentation using Fiji software (Schindelin et al, 2012). Briefly, z-stack digital micrographs were opened using Fiji and histogram equalization optimised image contrast. Sections were then condensed to a single image using the ImageJ maximum intensity projection command and scale bars were added.

2.2.5 Cerebellar nuclei: soma size analysis

In some sections the size distribution of neurons with β_1- or β_2-adrenoceptor IR in the cerebellar nuclei was measured. These sections were double labelled with anti-β-Tubulin III to selectively label neurons. From nuclear montages, multiple regions of 250 µm x 250 µm were selected at random and cropped. Histogram equalization was

performed on the cropped images and a condensed image was created using the maximum intensity projection command. Images were thresholded and made binary; the ‘Open’ operation was performed on the binary images to smooth objects and remove isolated pixels by eroding the perimeter of all signal positive (signal+) objects by one pixel and then dilating the resultant objects uniformly by one pixel. This procedure allowed the signal from IR somata to be isolated from signal localised to smaller cell processes. The object counter command was used to measure the surface areas of the remaining signal+ objects. The measurements of the counted/ measured objects were overlaid on to the corresponding micrographs of β_1, β_2 and β-Tubulin III IR. The measured sizes (μm^2) of all isolated β_1, β_2 and β-Tubulin III IR somata were recorded. Based on the observations in Uusisaari et al (2007) that the difference in average somatic surface area of smaller GABAergic and larger glutamatergic projection neurons is approximately 200 μm^2 we categorised the observed neurons into four categories of 200 μm^2 (0-200 μm^2, 201-400 μm^2, 401-600 μm^2, and 600 μm^2+).

2.3 Results

Immunohistochemistry was used to examine distribution of β_1-and β_2-adrenoceptors (β_1 and β_2) by cell type in the cerebellum of adult rats. This analysis was indicated by earlier behavioural experiments that had demonstrated important roles for β-adrenoceptor mediated NA signaling in the acquisition and/or consolidation of cerebellum-dependent learning tasks. Additionally, α_1-adrenoceptor distribution by cell type in the cerebellar cortex was investigated. Electrophysiological experiments had shown that α_1-adrenoceptor activation influences cerebellar cortical signaling but very little is currently known about the cellular distribution of α_1-adrenoceptor in the adult cerebellum.

2.3.1 The β_1-adrenoceptor is expressed in Purkinje cells throughout the cerebellar cortex

The cellular distribution of anti-β_1 IR is summarized in table 2.2. Anti-β_1 IR is present in large somata that form a monolayer throughout the cortex and in extensive processes in the molecular layer. The large size and monolayer arrangement of the β_1+ somata indicate they are likely to be PC somata. β_1+ processes continuous with the β_1+ somata are often seen (e.g. Fig. 2.3A arrowheads) so many of these processes are likely to be PC dendrites. Furthermore, the pattern of these β_1+ processes when viewed in coronal sections differs across regions of the cortex. In midline vermis the processes are in palisade arrangement (Fig. 2.3A and 2.3C) whilst in lateral vermis, hemispheral lobules or floccular regions, the processes become increasingly fan-like in appearance (Fig. 2.3B). This is the characteristic morphology of PC dendrites viewed in coronal sections. The orientation of the PC dendritic fan remains perpendicular to the long axis of its folium but the variation in foliar orientation with distance from the midline in coronal section means that the PC dendritic trees are seen in different planes across the sections. These morphological signs strongly suggest that β_1-adrenoceptors are expressed in Purkinje cells.

The β_1+ processes and somata also express calbindin (Fig. 2.3A) and Zebrin II (Fig. 2.3B and 2.3C) which are both PC specific marker proteins, substantiating the morphological observations. Anti-Zebrin II labels subsets of PCs across the cortex. In most regions of the cerebellum Zebrin II+ and Zebrin II- PCs are arranged in

longitudinally oriented stripes, whilst in other regions Zebrin II labels all PCs. Where Zebrin II is uniformly expressed, β_1 expression is present in all Zebrin II+ PCs (Fig. 2.3B), whilst in regions of heterogeneous Zebrin II expression β_1 immunoreactivity colocalises with Zebrin II+ PCs and additional expression is seen in putative Zebrin II- PCs (Fig. 2.3C). That β_1 expression is found in all PCs suggests a fundamental role for β_1-adrenoceptor mediated NA signalling independent of function relating to Zebrin II expression.

Antigen (N)	TUBB3 (4)	CALB1 (6)	PVALB (4)	GFAP (4)	S100B (4)
Colocalisation with β_1	Yes	Yes	Yes	No	No
Notes	Colocalisation with PC somata and dendrites, no other cell types	Colocalisation with PC somata and dendrites.	No colocalisation of ML IR with parvalbumin+ MLI. Colocalisation of PCL IR with PC somata	No colocalisation of ML IR with BGC processes	No colocalisation of PCL IR with BGC somata

Table 2.2: Antibodies tested for double immunolabelling with β_1-adrenoceptor antibody.

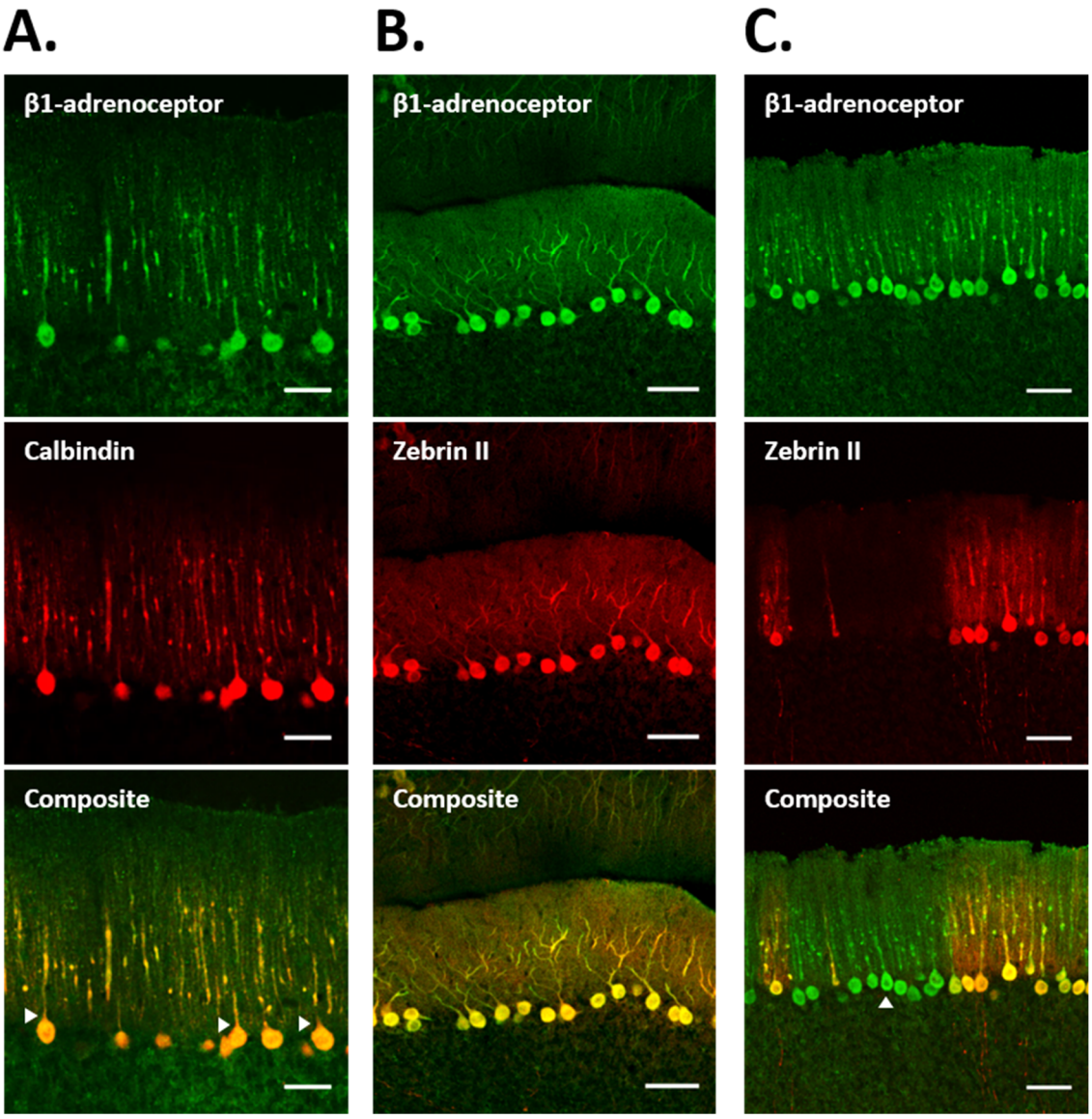


Fig. 2.3: β_1-adrenoceptor expression colocalises with Purkinje cell specific marker proteins Calbindin and Zebrin II. A) Calbindin and β_1-adrenoceptor colocalisation. β_1 IR colocalises with calbindin in the molecular layer and Purkinje cell layer, indicating that Purkinje cells express β_1. β_1+ processes can be seen to emerge from β_1+ somata (arrowheads). **B and C) Zebrin II and β_1-adrenoceptor colocalisation.** As with calbindin, complete colocalisation of β_1 and Zebrin II IR is observed in Purkinje cells. In regions of uniform Zebrin II expression full colocalisation is seen (B). In regions of striped Zebrin II expression, β_1 is expressed by all Zebrin II+ Purkinje and by putative Zebrin II- Purkinje cells (arrowhead in C). A-C: coronal sections. A, C: midline vermis, B: Flocculus. Scale bars: A: 50 µm, B and C: 120 µm

2.3.2 β_1-adrenceptors are not expressed by non-Purkinje neural cell types or by Bergmann glia in the cerebellar cortex

Some earlier studies suggesting β_1-adrenoceptor expression in the PCL lacked resolution sufficient to distinguish between PC and Bergmann glial cells (BGC) which are unique to the cerebellar cortex. BGC somata are present in the PCL and their processes extend through the ML, so β_1 immunoreactivity seen in both of these layers and previously presumed to be PC-related may, in fact, relate partly or exclusively to BGC.

Here, we saw that molecular layer β_1 IR does not colocalise with GFAP+ Bergmann glia processes (Fig. 2.4A and 2.4X). BGC somata in the PCL were defined by anti-S100β IR (Fig. 2.4B and 2.4Y) and again there was no colocalisation of β_1 and S100β expression. Visually S100β+ BGC somata are entirely distinct from the much larger β_1+ somata. Together these experiments indicate that β_1-adrenoceptors are not expressed in Bergmann glia somata or processes.

Parvalbumin is a marker for PCs and MLIs. Using anti-parvalbumin IHC we were able to distinguish MLI somata and PC dendrites in the ML based on their distinctive morphologies. As previously noted, β_1 IR colocalised to PC processes but no β_1 expression was observed on elements that could be morphologically defined as basket or stellate cell somata (arrowheads: Fig. 2.5B and 2.5Y). Anti-β-Tubulin III IHC labels structures in the GCL but did not colocalise with β_1 IR (Fig. 2.5C and 2.5Z). Cells whose morphology identified them as putative GoCs can also be seen. Their radiating dendritic arborisations did not show β_1 expression (black arrowhead: Fig. 2.5C and 2.5Z). The Lugaro cell, identifiable by a prominent dendrite extending from its somata (arrow: Fig. 2.5C and 2.5Z) also does not appear to express β_1. Additionally, β-Tubulin III labelled cells with morphologies identifiable as stellate and basket cells (white arrowhead: Fig. 2.5C and 2.5Z) had no colocalisation with β_1. This finding, based on morphological criteria, confirms the findings seen with Parvalbumin identification of the MLIs. Finally, β_1+ cells did not express calretinin, confirming that β_1-adrenoceptors are neither expressed by LGCs (arrowhead: Fig. 2.5A and 2.5X) nor by UBCs (arrow: Fig. 2.5A and 2.5X).

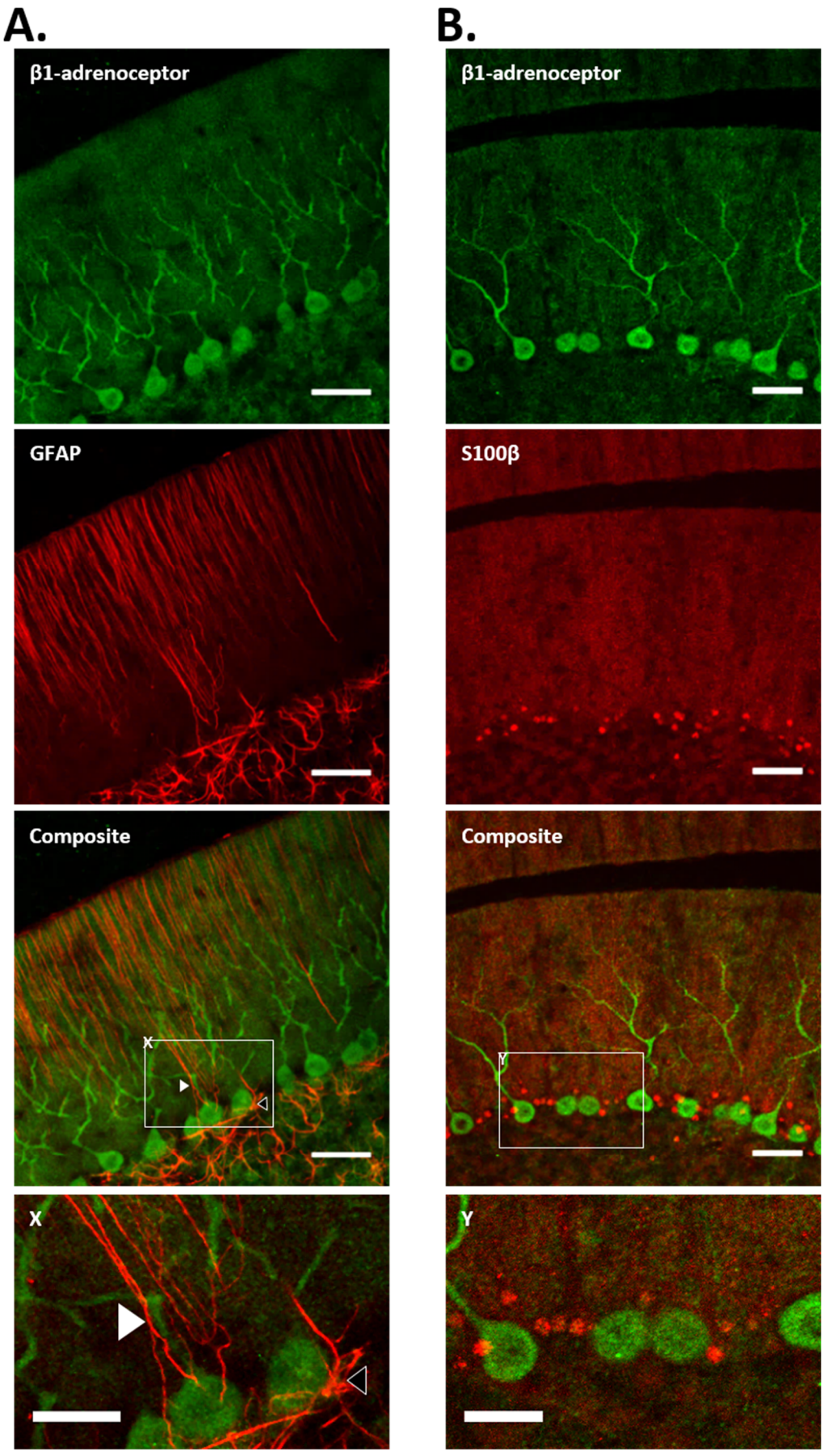


Fig. 2.4 - Legend overleaf.

Fig. 2.4: β_1-adrenoceptor expression does not colocalise with the glial cell marker proteins GFAP and S100B. A and X) GFAP and β_1-adrenoceptor colocalisation. GFAP+ BGC radial processes throughout the ML do not express the β_1-adrenoceptor. This is particularly clear in A. In this section plane for lobule HVI, β_1-adrenoceptor+ processes are seen in the fan arrangement typical of Purkinje cell dendrites. In contrast GFAP+ Bergmann glia processes are in a radial arrangement. Bergmann glia radial processes in the molecular layer are visually distinct from β_1+ processes (A and X: white arrowhead) and GFAP+ astrocytes do not colocalise with β_1+ somata in the Purkinje cell layer (A and X: Black arrowhead) **B and Y) S100B and β_1-adrenoceptor colocalisation.** In the Purkinje cell layer S100B+ Bergmann glia somata do not express β_1-adrenoceptors, Bergmann glia somata are entirely distinct in size from β_1-adrenoceptor+ somata, as can be seen in Y. A-B: coronal sections. A: hemispheral lobules, B: paraflocculus. Scale bars: A and B: 50 µm, X and Y: 25 µm.

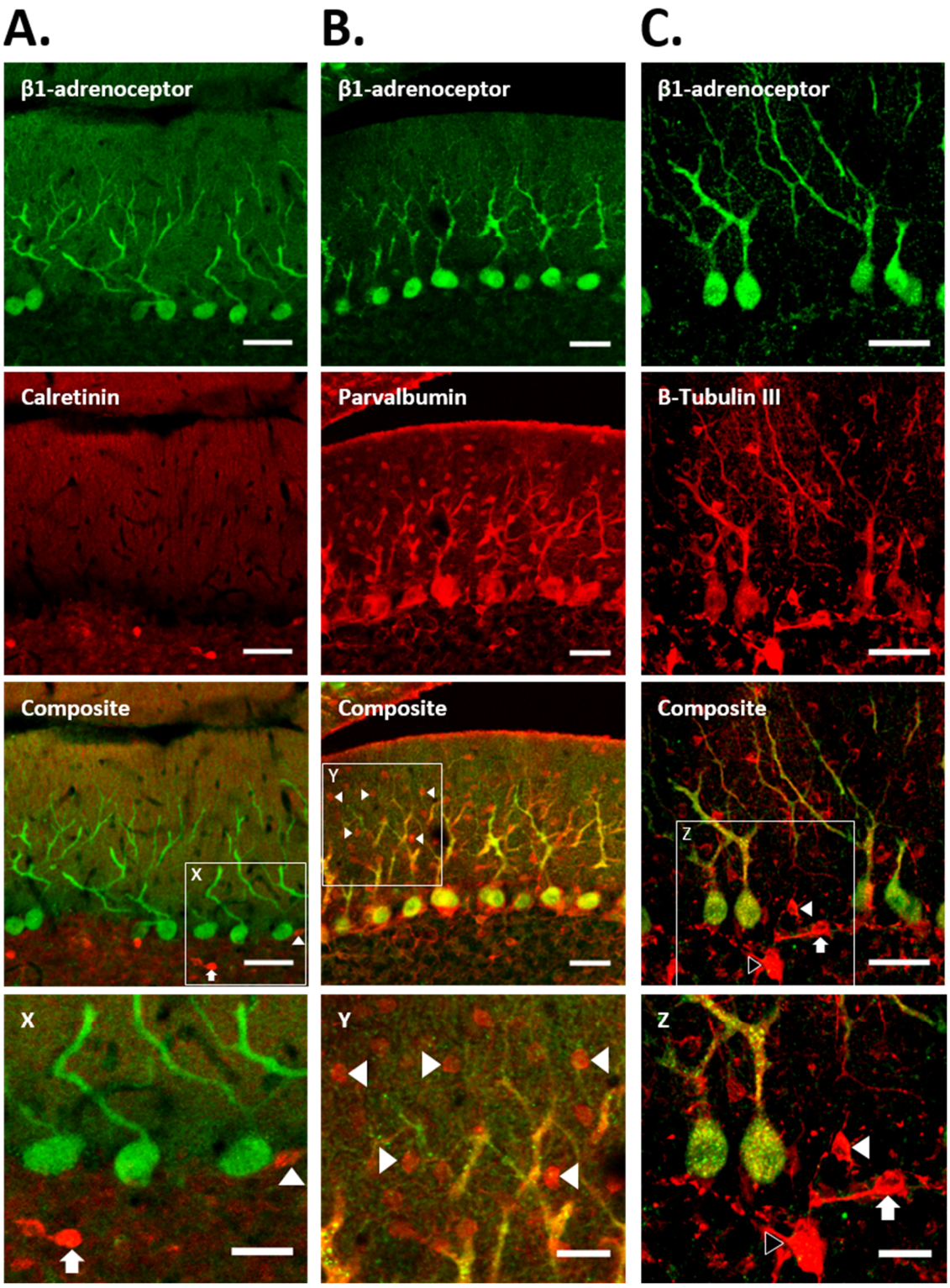


Fig. 2.5 – Legend overleaf.

Fig. 2.5: β_1-adrenoceptor expression does not colocalise with markers for other neural cell types in the cerebellar cortex. A and X) Calretinin and β_1-adrenoceptor colocalisation. No colocalisation of calretinin+ neural elements and β_1 expression is observed in the GCL elements including UBCs (A and X: arrow) and LGCs (A and X: arrowhead). **B) Parvalbumin and β_1-adrenoceptor colocalisation.** β_1 expression colocalised with parvalbumin+ PC dendrites and somata but no β_1 expression was observed in morphologically distinct parvalbumin+ MLIs (B and Y: arrowheads) **C) β-Tubulin III and β_1-adrenoceptor colocalisation.** As with parvalbumin β_1 expression colocalised with β-Tubulin III+ PC dendrites and somata but did not colocalise with MLIs (B and Z: white arrowhead). Additionally, in the GCL, β_1 expression was absent from large interneuron somata (B and Z: putative GoC: black arrowhead, putative LGC: arrow). A-C: coronal sections. A, C: paraflocculus, B: hemispheral lobule. Scale bars: A, B and C: 50 µm, X, Y and Z: 25 µm

2.3.3 β_2-adrenoceptor expression is distinct from that of β_1-adrenoceptor and is largely restricted to Bergmann Glia in the cerebellar cortex

As with β_1 expression, β_2 expression is present in both the PCL and ML (Fig. 2.6A and 2.6C). However, the two patterns of immunoreactivity are qualitatively distinct. β_2-immunoreactivity in the molecular layer is seen as thin radial processes with the appearance of Bergmann glia processes. Triple immunolabelling of anti- β_2 and anti-S100β / anti-GFAP showed colocalisation of β_2+ elements with Bergmann glia somata (white arrows: Figure 2.6A and 2.6Y), processes (white arrowheads: Fig 2.6A and 2.6X) and end feet on the pial surface of the cerebellum (black arrows: Fig.2.6A). β_2 expression in the Purkinje cell layer appears to localise to an intermediate region where the Bergmann glia process emerge from their somata (arrowheads: Fig. 2.6B and 2.6Z). Taken together, these findings strongly suggest that β_2 expression is restricted to Bergmann glia.

Antigen (N)	TUBB3 (4)	CALB1 (6)	GFAP (4)	S100B (4)
Colocalisation with β_2	No	No	Yes	Yes
Notes	No strong colocalisation with any neural cell types.	No colocalisation of PCL IR. Distinct patterns of IR in ML.	Similar pattern of IR in ML, significant IR colocalisation in ML.	Colocalsation of PCL IR with BGC somata

Table 2.3: Antibodies tested for double immunolabelling with β_2-adrenoceptor antibody.

2.3.4 Cerebellar cortical β_2-adrenoceptor expression is low to absent in neural elements revealed by anti-β-tubulin III

Substantial regions of β_2 immunoreactivity colocalise with GFAP and S100β but β_2 immunoreactivity does not fully overlap with GFAP immunoreactive BGC processes in the ML (black arrowheads: Fig. 2.6X). This may indicate that β_2-adrenoceptors are additionally expressed by other cell types, in sub-cellular compartments in close association with BGC processes. To determine whether some of the β_2+ elements in the molecular layer are neural we double immunolabelled sections with anti-β-tubulin III (Fig. 2.6C). However, no colocalisation of β_2 and β-tubulin III immunoreactivity was observed in the PCL or ML. This was particularly clear in the regions where PC dendritic trees are seen in fan arrangement while β_2 immunoreactivity exhibited the radial pattern characteristic of BGC processes (Fig. 2.6C). β_2-adrenoceptor expression was not present in PC somata (* in Fig. 2.6C) or processes (white arrowheads: Fig. 2.6C), large interneurons in the GCL (black arrowheads: Fig. 2.6C) and MLIs (arrow: Fig. 2.6C).

There is a weak possibility that the slightly shifted registration of anti-GFAP and anti-β_2 expression patterns may arise because the two proteins are expressed in different subcellular locations within the same cell. GFAP is a structural protein and anti-GFAP reveals only the central spine of the Bergmann glia processes, which are in fact a highly ramified structure with lateral “bristle-like” appendages that ensheath the PF-PC synapses. So it may be necessary to use electron microscopy or an ultra-high resolution fluorescence microscopy technique unambiguously to define whether β_2-expression is restricted to BGC in the ML or is partially expressed in subcellular compartments of neural cell types in close apposition to BGC processes (e.g. PF terminals or PC dendritic spines).

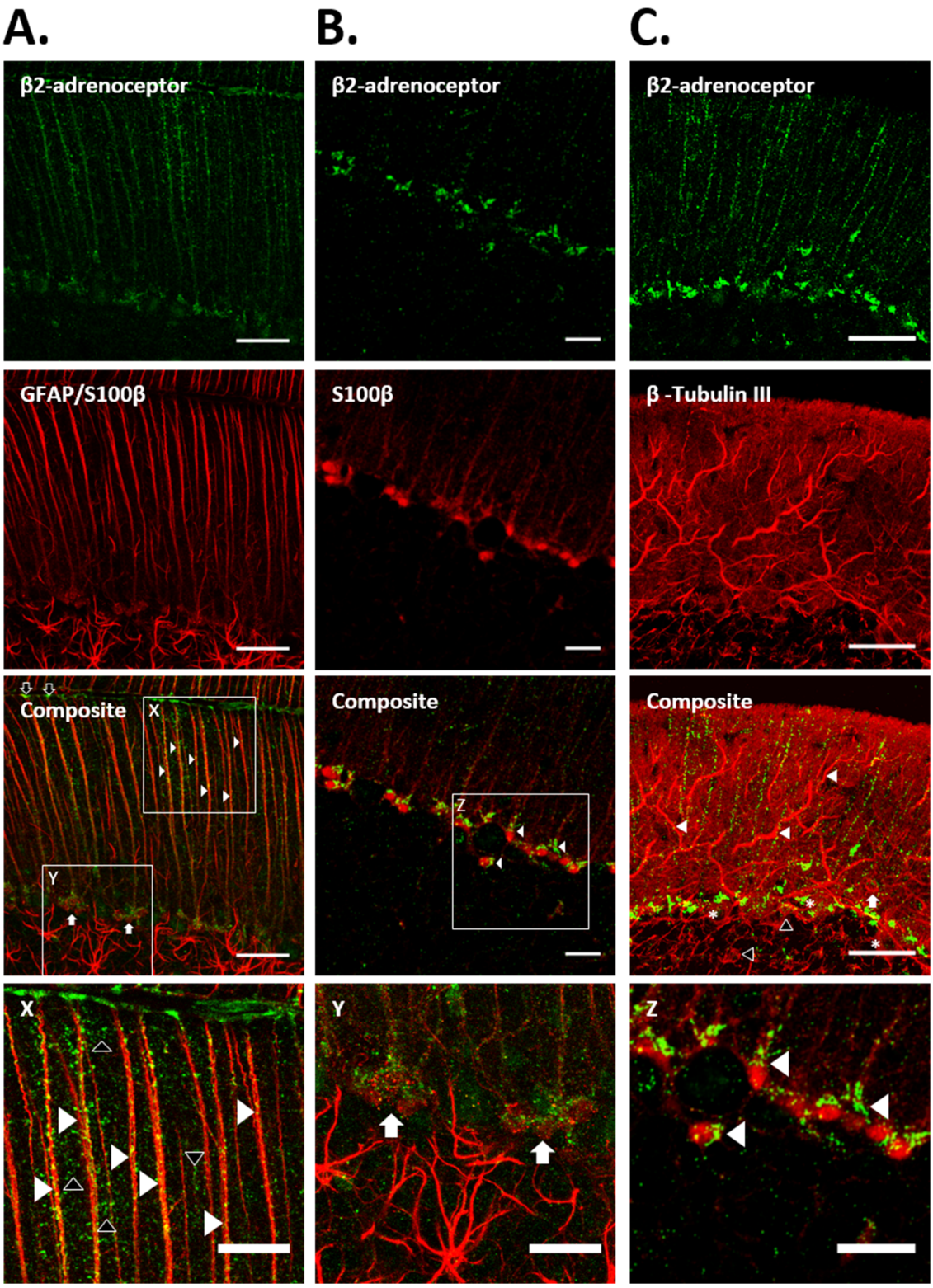


Fig. 2.6 – Legend overleaf.

Fig. 2.6: β_2-adrenoceptor is predominantly expressed by Bergmann glia cells in the cerebellar cortex and does not appear to be expressed by neurons. A, X and Y) GFAP/ S100β and β_2-adrenoceptor colocalisation. When anti-S100β and anti-GFAP are used in combination to label both the somata and processes of the Bergmann glia, colocalisation of S100β and β_2 expression is observed at BGC somata in the PCL (A and Y: arrows) and colocalisation of GFAP and β_2 is observed at BGC processes in the ML (A and X: white arrowheads) and the end feet of the processes at the pial surface (A: black arrows). However, in some regions of the molecular layer β_2 expression can be in very close proximity to BGC processes but it does not appear exactly to colocalise (X: black arrowheads). **B and Z) S100β and β_2-adrenoceptor colocalisation.** In the PCL β_2 expression is clustered in a pattern similar to the clustering of BGC somata (B and Z: arrowheads). Closer examination of the β_2 expression in the PCL indicates that it is concentrated adjacent to the distal region of the BGC process (Z: arrowheads). **C) β-Tubulin III and β_2-adrenoceptor colocalisation.** β_2 expression is not seen on β-Tubulin III+ large interneurons in the GCL (black arrowheads), PC somata (*) or dendrites (white arrowheads) or MLIs (arrow). Indicating that β_2 protein is not present in the somata or major processes of these cells. A-C: coronal sections. A-B: vermis lobules, C: hemispheral lobule. Scale bars: A and C: 50 μm, B, X, Y and Z: 25 μm

2.3.5 Distinct distributions of β_1- and β_2-adrenoceptor immunoreactive neurons in the cerebellar nuclei

Both β_1- and β_2-adrenoceptors are expressed in all cerebellar nuclei (β_1: Fig. 2.9A and 2.9X. β_2: 2.9B, 2.9Z and 2.9Y). Expression of both β-adrenoceptors was predominantly somatic in nature. Because of a lack of antibodies that can distinguish cerebellar nuclear cell types at the somata we were unable to define, in detail, the cell type expression of the β-adrenoceptors using immunohistochemistry (Uusisaari and De Schutter, 2011 and see discussion). However, by measuring the cross sectional areas of β-tubulin III+ somata, including those that coexpress β_1- or β_2-adrenoceptors we were able to define the range of cell types (Fig. 2.7. N: 4) in the nuclei by size and plot the distribution of β-adrenoceptor+ cells within the general population.

Expression of both β-adrenoceptor types was observed across the range of cell sizes. But a lower proportion of somata in the small size range are β_1 IR in comparison to the proportion of small somata that are β_2 IR and to the proportion of intermediate and large cells that are β_1 IR, suggesting the β_1-adrenoceptor is more prevalent on the intermediate to large projection neurons than small interneurons (Fig. 2.8). This is reflected by observation that many of the smallest β-Tubulin-III IR somata did not colocalise with β_1 IR (e.g. arrowheads in Fig. 2.9A and 2.9X indicate two small neurons that do not express β_1. Table 3.4), although smaller β_1+ cells are present.

In contrast, β_2 IR was much more uniformly present and was seen in high proportions of cells in all size ranges (Fig. 2.8) indicating that β_2-adrenoceptors are probably expressed uniformly and by a large proportion of all cell types in the cerebellar nuclei. Small β_2 IR somata, which are putative local interneurons were more numerous than small β_1 IR somata (e.g. arrowheads in Fig. 2.9B, 2.9Z and 2.9Y) and more numerous than β_2 IR somata of a range of sizes (Fig. 2.9B, 2.9Z and 2.9Y).

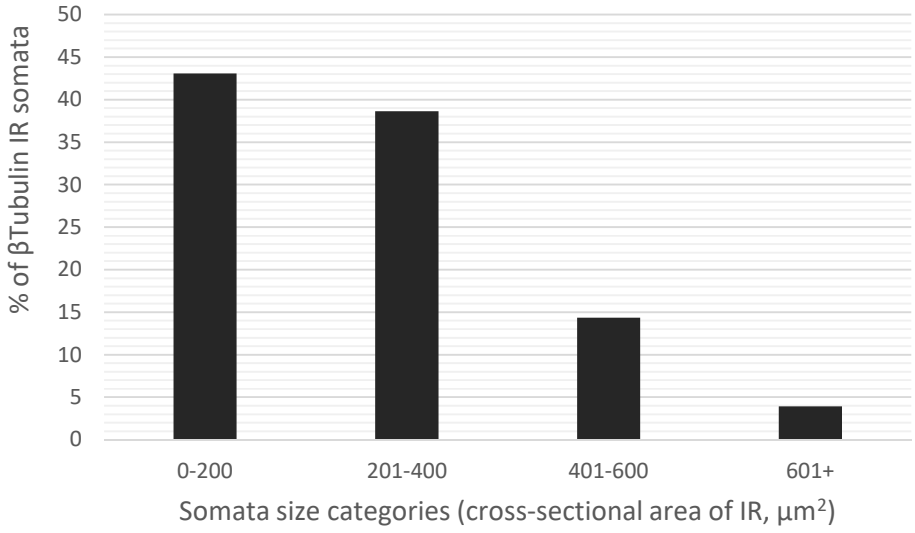


Fig. 2.7: Percentage of β-Tubulin III IR somata that populate each size category of cerebellar nuclear neuron. 0-200 µm^2: ~43%; 201-400 µm^2: ~38%; 401-600 µm^2: ~14% and 600+ µm^2: ~4

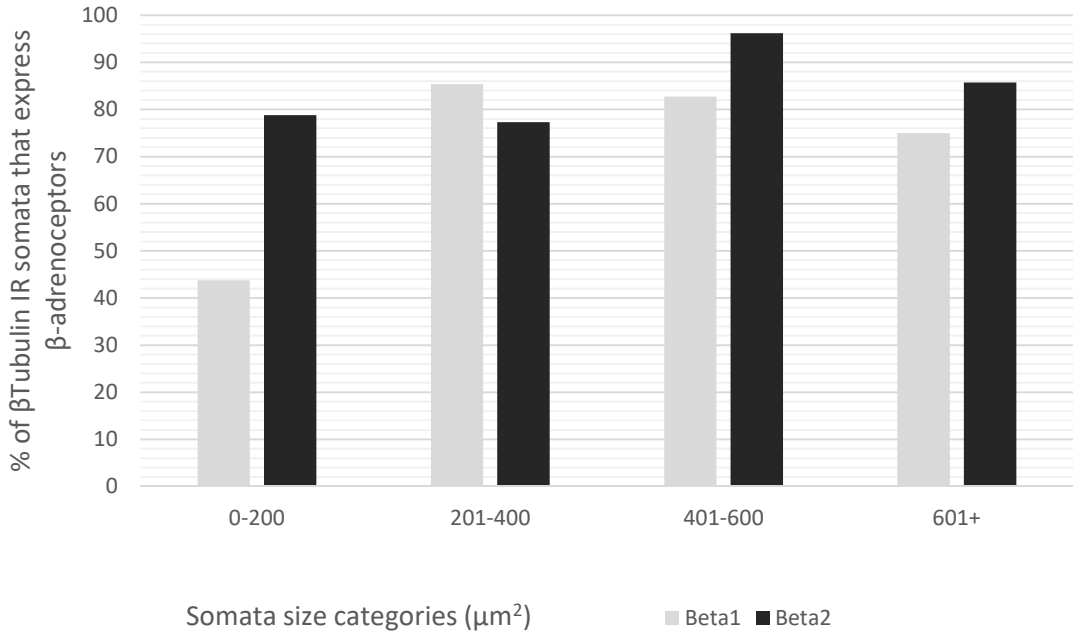


Fig. 2.8: Percentage of β-Tubulin III IR somata that are β-adrenoceptor IR in each cell size category. A: Percentage of β-Tubulin III IR somata that are β_1 IR. 0-200 µm^2: ~43%; 201-400 µm^2: ~85%; 401-600 µm^2: ~83% and 600+ µm^2: ~75%. **B:** Percentage of β-Tubulin III IR somata that are β_2 IR. 0-200 µm^2: ~79%; 201-400 µm^2: ~77%; 401-600 µm^2: ~96% and 600+ µm^2: ~85%.

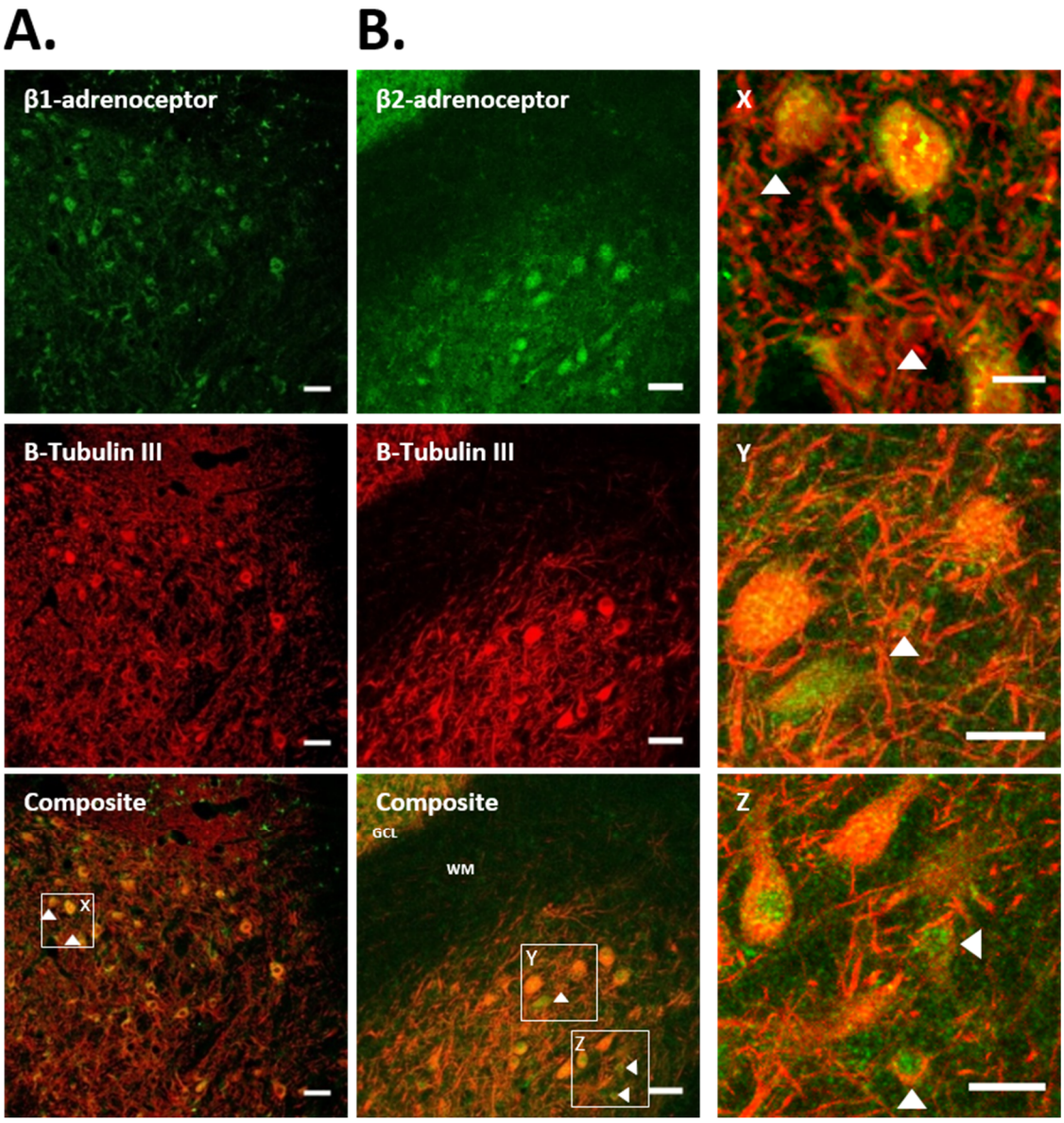


Fig. 2.9: β_1- and β_2-adrenoceptor expression is present throughout the cerebellar nuclei. A) β_1-adrenoceptor expression in the cerebellar nuclei. β_1+ cells are present throughout the cerebellar nuclei. Not all observed β-Tubulin III+ cells express β_1, particularly the smaller neurons (A and X: arrowheads). **B) β_2-adrenoceptor expression in the cerebellar nuclei.** β_2+ cells are present throughout the cerebellar nuclei. β_2 expression appears more uniform with many large and small neurons exhibiting β_2 expression, however the proportion of β_2+ IR small neurons is smaller than the proportion of β_1+ IR small neurons (B, Y and Z: arrowheads). A-B: coronal sections. Scale bars: A and B: 50 µm, X, Y and Z: 25 µm. WM: White matter. GCL: Granule cell layer

2.3.6 α_1-adrenoceptor expression is expressed by several neural cell types in the cerebellar cortex

Immunoreactivity for α_1-adrenoceptor (α_1) expression was present in the PCL, small somata throughout the ML and in the GCL on large, round or polygonal somata (Fig. 2.10A, B and C and 2.11A and B). Antibody combinations are summarised in table 2.4. Double immunolabelling using calbindin confirmed that α_1 IR in PCL was on PC somata (Fig.2.10A). In the ML the small α_1+ somata colocalised with β-Tubulin III suggesting it is expressed by MLIs (Fig. 2.10B). Within the GCL, there are several categories of inhibitory cells that may express α_1 IR; Golgi cells, Lugaro cells and unipolar brush cells (UBC's). Across the vestibular cerebellar regions, where UBS can be found, UBC's were ruled out based on the morphology of the observed α_1+ cells. UBC's have a small circular soma with a single, distinctive 'brush' like dendrite close to the cell body, whilst the processes observed on the α_1 IR somata were relatively large and lacked any such distinctive outgrowths. Thus Golgi cells and Lugaro cells were candidates for α_1 expression. Anti-calretinin is a marker of both UBC's and Lugaro cells (Geurts et al 2001; 2002) and in sections double immunolabelled for anti-α_1 and anti-calretinin, there were no double labelled cells (Fig. 2.11B) confirming the absence of α_1 IR in UBC's and Lugaro cells. Golgi cells were identified using anti-$mGlu_2$ (Geurts et al 2001; 2002) and there was clear double immunolabelling of somata in the GCL (Fig. 2.10C) indicating that α_1 expression in the GCL is found specifically on Golgi cells. Finally, α_1 does not appear to colocalise with GFAP indicating it has an entirely neural expression pattern (Fig. 2.11A)

Antigen (N)	TUBB3 (4)	CALB1 (6)	GFAP (4)	S100B (4)
Colocalisation with α_1	No	No	Yes	Yes
Notes	No strong colocalisation with any neural cell types.	No colocalisation of PCL IR. Distinct patterns of IR in ML.	Similar pattern of IR in ML, significant IR colocalisation in ML.	Colocalsation of PCL IR with BGC somata

Table 2.4: Antibodies tested for double immunolabelling with α_1-adrenoceptor antibody.

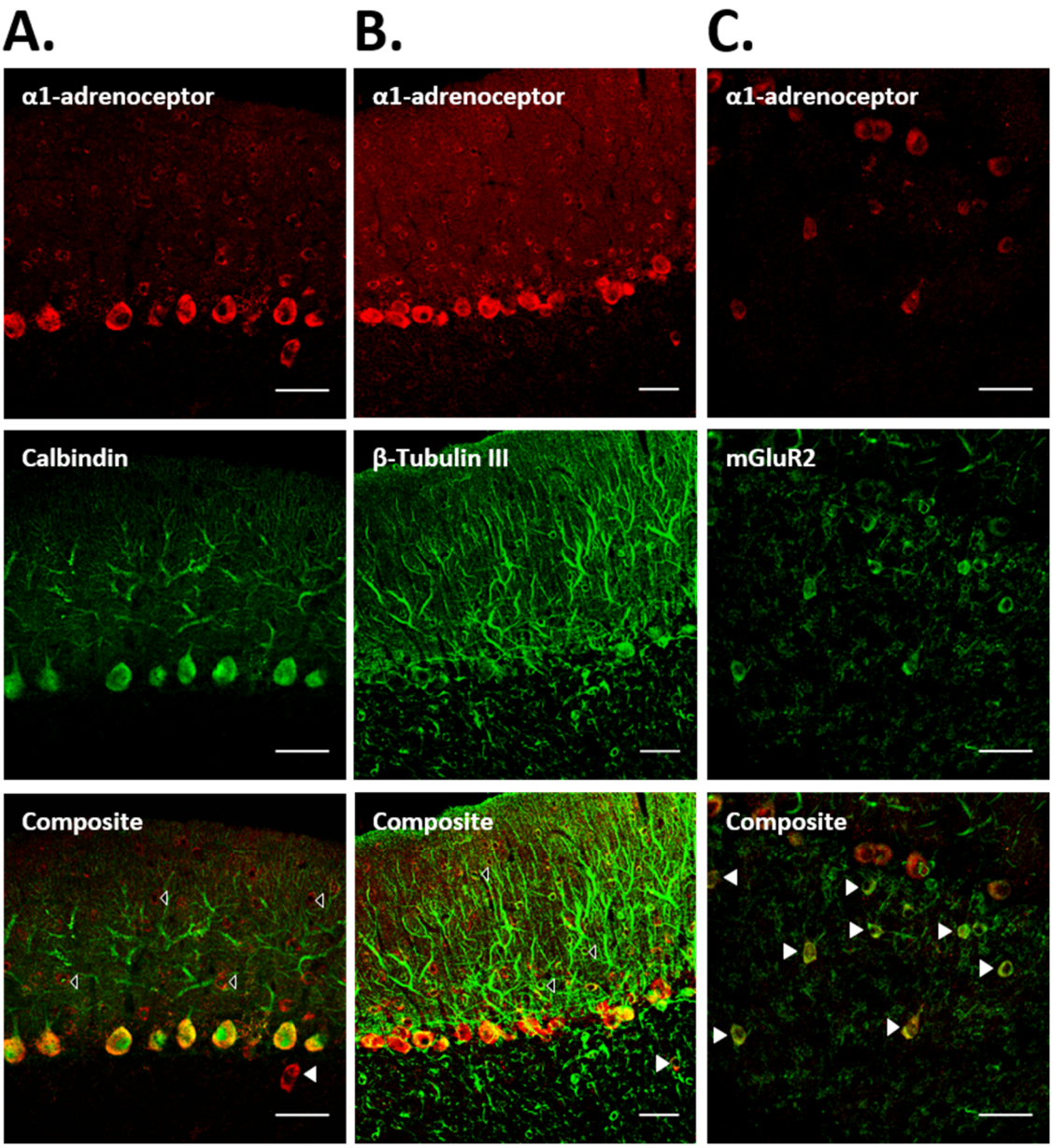


Fig. 2.10: α_1-adrenoceptor expression is present in PC somata, molecular layer interneurons and $mGlu_2$+ Golgi cells. A) α_1-adrenoceptor and Calbindin colocalisation. α_1+ cells are present in all three layers of the cerebellar cortex. In the ML there is α_1 IR in small somata that does not colocalise with calbindin, which suggests the cells are MLIs (A: open arrowheads). PC somata in the PCL are α_1+ but large somata in the GCL, possible GoCs or LGCs, are not (A: arrowhead) **B) α_1-adrenoceptor and β-Tubulin III colocalisation.** The PC somata are α_1+ cells throughout the PCL. There is α_1 IR in large somata in the GCL which colocalise with β-Tubulin III IR (B: arrowhead. These cells are to be of the same type that did not express calbindin). Finally, the α_1+ elements in the ML do colocalise with β-Tubulin III suggesting they are MLIs (B: open arrowheads). **C) α_1-adrenoceptor and $mGlu_2$ colocalisation.** In the GCL α_1+ cells colocalise with $mGlu_2$ (C: arrowheads) which confirms that GoCs express α_1-adrenoceptors. A-C: coronal sections. Scale bars: 50 µm

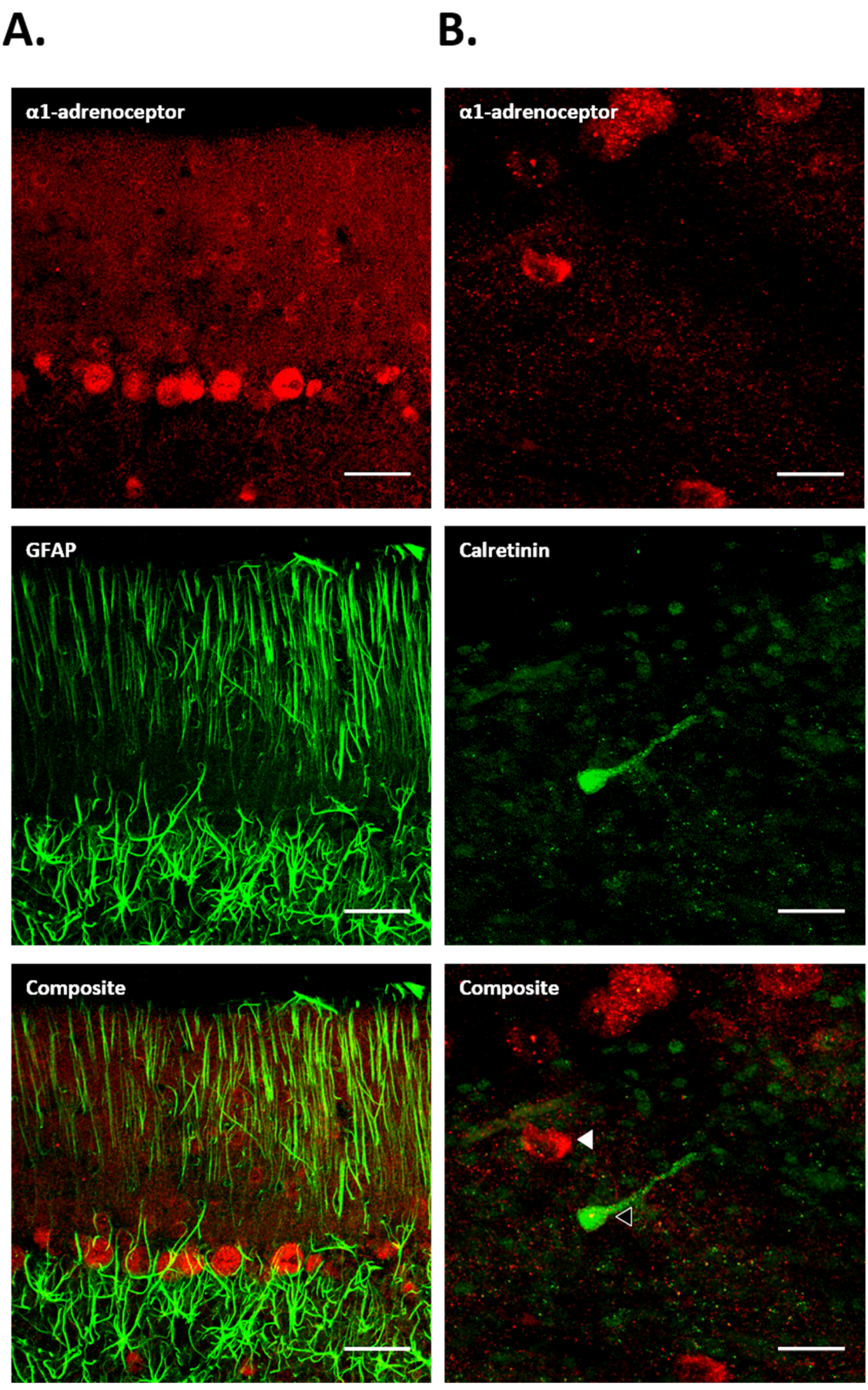


Fig 2.11 – Legend overleaf.

Fig. 2.11: α_1-adrenoceptor expression does not colocalise with GFAP+ glial elements or calretinin+ Lugaro cells. A) α_1-adrenoceptor and GFAP colocalisation. In the ML and GCL no α_1+ elements colocalise with GFAP indicating that α_1-adrenoceptors are not expressed by BGC or astrocytes in the GCL. **B) α_1-adrenoceptor and β-Tubulin III colocalisation.** Similarly the PC somata are α_1+ cells throughout the PCL. There is a large α_1+ somata in the GCL which does colocalise β-Tubulin III (B: arrowhead. This is likely to be of the same type that did not colocalise with calbindin). Finally, the α_1+ elements in the ML do colocalise with β-Tubulin III suggesting they are MLIs (B: open arrowheads). **C) α_1-adrenoceptor and calretinin colocalisation.** In the GCL α_1+ cells (B: arrowhead) do not colocalise with calretinin+ LGCs (B: open arrowhead) indicating the GCL expression of α_1 is probably restricted to GoCs. A-B: coronal sections. A: vermis lobule, B: Lobule X. Scale bars: A: 50 μm and B: 25 μm

2.4 Discussion

These studies have analysed the anatomical distribution of key receptors involved in noradrenergic signalling in the cerebellum. The cellular distribution of the β_1-, β_2- and α_1-adrenoceptors was revealed using receptor protein IHC. Understanding the distribution by cell type of the receptors that mediate NA signalling in the cerebellum is an important first step in understanding the mechanisms underpinning previous behavioural results and in the interpretation of future studies that will use selective manipulations of adrenoceptor function.

β_1-adrenoceptor IR was localized to PC somata and dendrites. In contrast, β_2-adrenoceptor IR was found on radial processes through the molecular layer and throughout the PCL. This expression was determined to be significant in BGC somata and processes after examining colocalisation with various cell markers. α_1-adrenoceptor IR was localised to a range of neural types: PC and MLI somata and $mGlu_2$ expressing Golgi cells.

The observed distribution of β-adrenoceptor expression provides an anatomical foundation for experimental observations that application of β-adrenoceptor antagonists impairs memory acquisition for various cerebellum-dependent learning tasks and particularly the consolidation of classical conditioning of the NMR/EBR (Paredes et al, 2009; Kellett and Yeo, 2007).

2.4.1 β_1-adrenoceptor distribution – implications for NA function in cerebellar learning

The localization of β_1-adrenoceptors on PCs is consistent with the hypothesis that NA acts in combination with PF and CF activity as a ‘third signal’ to the PC to facilitate memory consolidation processes (Gilbert, 1975). A series of *in vivo* electrophysiological studies have shown that application of NA to the cerebellum potentiates the inhibitory effect of GABAergic signalling on PCs (Moises et al, 1979), an effect mediated by activation of the β_1-adrenoceptor postsynaptically on PCs (Yeh and Woodward, 1983 and Cheun and Yeh, 1992). The present experiments provide the anatomical substrate for these electrophysiological observations.

The nature and location of plasticity that underpins cerebellar-dependant learning in general and in NMR/EBR in particular remains controversial but current evidence indicates an essential cortical mechanism (Kellett et al, 2010). Electrophysiological evidence is consistent with the suggestion that most, if not all of the underlying plasticity is at the Purkinje cell (Jirenhed et al, 2007; Jirenhed and Hesslow, 2015). So it may be that the actions of the β-adrenoceptor antagonists propranolol (Cartford et al; 2004a; Paredes et al, 2009) or atenolol (Kellett and Yeo, 2007) to impair acquisition or consolidation of EBR and NMR conditioning, respectively, are mediated solely via β_1-adrenoceptors on Purkinje cells. This is particularly likely for the effects of atenolol on consolidation (Kellett and Yeo, 2007) because atenolol is a relatively selective antagonist of β_1-adrenoceptors.

2.4.2 β_2-adrenoceptor distribution – the possibility of a glial signalling component of NA function in the cerebellum

The observation that Bergmann glia cells express the β_2-adrenoceptor subtype indicates a novel neuron to glia signalling pathway. Although glial cells were traditionally characterised as non-signalling support cells it is increasingly clear they actively contribute to neural signalling and plasticity processes in many brain circuits.

Bergmann glia processes closely ensheath PF and CF synapses on Purkinje cells (Watanabe, 2002; Matsui and Jahr, 2004; Matsui et al, 2005). The ensheathing processes are known as lateral appendages; they express calcium permeable membrane-bound AMPA receptors (Matsui and Jahr, 2004; Matsui et al, 2005) and they are thought to be critical for mediating synaptic transmission to PCs (Saab et al, 2012). Stimulation of PFs *in vitro* causes a significant calcium influx in BGCs, an effect potentially mediated by AMPA receptors expressed on the lateral appendages or by metabotropic glutamate and purinergic receptors also expressed by BGCs (Beierlein and Regehr, 2006; Piet and Jahr, 2007) or by nitric oxide signalling (Matyash et al, 2001). How might NA modulation of BGC activity contribute to memory consolidation? Wang et al (2012) showed that BGC calcium influx modulates PC activity through a calcium mediated regulation of extracellular potassium and Sasaki et al (2012) showed optogenetic activation of BGCs *in vivo* or *in vitro* leads to glutamate release by BGCs which activates PCs via AMPA receptor activation and induces LTD of the PF-PC synapse *in vitro* via activation of extrasynaptic $mGlu_1$ receptors. In sum, these studies suggest that through activation by neurotransmitter signalling, the BGC is well placed

to modulate cerebellar cortical processing and provides a mechanism by which β_2-adrenoceptor mediated signalling influences activity in the cerebellar cortex.

In relation to motor behaviour, Nimmerjahn et al (2009) found that BGC calcium transients occur across networks of hundreds of BGCs simultaneously during locomotor behaviour and that they are driven by glutamatergic transmission. Paukert et al (2014) extended these findings to show that BGC calcium elevation correlates not just with locomotion but general arousal and, again, that the effect is modulated by glutamatergic signalling. The calcium transients were attenuated by glutamatergic antagonists and, importantly for the studies reported here, these transients were entirely blocked by α_1-adrenoceptor antagonists or by chemical depletion of NA. The work confirms an important role of NA signalling in inducing BGC activation during motor behaviour and arousal. In adult mice an induced knockout of AMPAR receptors in BGC produced a disruption of EBR conditioning that correlated with the loss of lateral appendages of the BGC and there are no corresponding deficits in UR performance (Saab et al, 2012). The findings suggest that β_2-adrenoceptor signalling on BGC is important for long-term memory mechanisms related to motor learning in addition to short-term maintenance of cerebellar physiology.

Importantly, the differences in β_1- and β_2-adrenoceptor distribution reported here underline the importance of using specific ligands selectively to manipulate signalling mediated by the two adrenoceptors during cerebellum-dependent learning tasks. NA signalling via Purkinje cells and Bergmann glia may contribute differently to memory related processes and these contributions may be tested by local application of β_1-and β_2-adrenoceptor antagonists. The anatomical observations reported here provide a framework for interpreting the results of these future experiments.

2.4.3 α_1-adrenoceptor distribution – implications for NA function in the cerebellum

The observation that α_1-adrenoceptors are expressed by PCs, MLIs and GOCs establishes the probable cells of origin for the IR observed previously in all three layers of rat cerebellar cortex in IHC studies that used lower resolution imaging methods (Acosta-Martinez et al, 1999; Papay et al, 2004; 2006). The Golgi cell performs a critical function in gating MF input to the granule cell layer and shaping the timing and

spatial distribution of GC signalling (e.g. Marr, 1969; D'Angelo et al, 2013). The α_1-adrenoceptor is therefore well placed to modulate PF and MLI signalling to the Purkinje cell. However, to date this is the first anatomical observation of α_1-adrenoceptor expression by the Golgi cell and no electrophysiology study has identified α_1-adrenoceptor mediated effects on Golgi cell activity, probably because most electrophysiological studies of noradrenergic signalling in the cerebellum have focused on PC activity because the PC is most accessible for recording. This observation is important in gaining a better understanding of the noradrenergic system in the cerebellar cortical circuit and in interpreting the effects of pharmacological manipulation. Herold et al (2005) and Hirono and Obata (2006) observed α_1-adrenoceptor mediated potentiation of spontaneous and evoked inhibition of Purkinje cells in vitro and suggested that the inhibition is mediated by activation of α_1-adrenoceptors on presynaptic inhibitory neuron terminals and somata. The observation of α_1-adrenoceptor IR on MLIs here provides anatomical support for this reported *in vitro* electrophysiology.

Previous electrophysiological studies (Herold et al, 2005; Hirono and Obata, 2006) did not observe any α_1-adrenoceptor mediated effect on PC activity but here we observed expression of this receptor on PCs. One possibility for the apparent inconsistency between the anatomical data presented here and previous electrophysiological studies is an artefact of the experimental conditions of the electrophysiology studies, specifically, the α_1-adrenoceptors expressed by PCs may not be functional or responsive under the experimental conditions used by Herold et al (2005) and Hirono and Obata (2006). An example of this phenomena comes from Di Mauro et al (2003; 2013). When Di Mauro et al (2003; 2013) applied NA to cerebellar nuclei neurons they observed an inhibitory effect on neurons in the FN and PIN but an excitatory effect in the AIN. However, when they used sub-type specific agonists they observed both inhibitory and excitatory effects in all three nuclear compartments, mediated by the α_2- and β-adrenoceptors respectively. Thus, though both subtypes are expressed in all nuclear compartment but under the specific experimental protocol used by Di Mauro et al (2003; 2013) α_2-adrenoceptors are the dominant functional receptor in response to NA in the FN and PIN and the β-adrenoceptors are dominant in the AIN.

2.4.4 Differences in the adrenoceptor distribution revealed by immunohistochemistry and suggested by previous electrophysiology studies

In addition to the observations of Herold et al (2005) and Hirono and Obata (2006) several previous *in vitro* investigations had observed a presynaptic β-adrenoceptor mediated potentiation of inhibitory drive on PCs (Kondo and Marty, 1998, Mitoma and Konishi, 1999, Saitow et al, 2000a, b; Saitow et al, 2005), which Saitow et al (2000a, b) suggest is mediated by action of NA on the β_2-adrenoceptor. However, in the current anatomical study we found no β_1- or β_2-adrenoceptor immunoreactivity on basket or stellate cells. There are two possibilities as to why this discrepancy may arise, it may be because the antibody used here may not reveal expression of the target receptor on all cells, in this case the MLIs. Another reason for the discrepancy may be because the *in vitro* studies used cerebellar tissue from juvenile/neonate rats or mice, whilst the current studies examined protein distribution in the adult rat cerebellum. Monoaminergic receptor expression levels have been shown to change significantly during the early postnatal period before settling into adult expression patterns many weeks postnatal; the most significant changes in expression level occur during the first four weeks of life in the rat (Murrin et al, 2007 also see Oostland et al, 2011; 2013; 2014 discussed in chapter 1 section 1.5.2). This is the same developmental stage of animals used in the *in vitro* studies discussed. This emphasizes the importance of using multiple methods in studying any phenomenon and particularly emphasizes the importance of examining adrenoceptor distribution in adult animals for interpretation of the behavioural effects of manipulating NA signalling in adult animals.

2.4.4 β-adrenoceptors in the cerebellar nuclei: a widespread population

The observation that both β_1- and β_2 -adrenoceptor subtypes are expressed in all cerebellar nuclei reveals that both types potentially mediate NA influence on cerebellar function. This is consistent with evidence from *in vivo* recordings from anaesthetised rats by Gould et al (1997) who observed a potentiation of the effect of GABA on cerebellar nuclei neurons after application of a β-adrenoceptor agonist and from Di Mauro et al (2003; 2013), who observed differential modulation of neurons in different cerebellar nuclei. Additionally our observation provides insight into the findings of Paredes et al (2009) who reported that post-training, cerebellar infusions of the β-adrenoceptor propranolol abolished consolidation even when the infusion was delayed by two hours, a time delay inconsistent with the same authors' observation that NA

levels in the cerebellar cortex were normalised by 80-90 minutes post-training. The reported sensitivity beyond the time window of learning-related cortical NA signalling could relate to spread of the large volume infusions of lipophilic propranolol to the underlying cerebellar nuclei, where a different temporal window for learning-related NA signalling might operate.

The distribution of sizes of somata expressing β-adrenoceptors indicates that β_2-adrenoceptors are expressed in a high proportion of all neurons types in the cerebellar nuclei, as defined by soma size. In contrast β_1 IR is present in a much higher proportion of the intermediate and large nuclear somata than small, indicating a bias towards expression of the β_1-adrenoceptor by the intermediate and large neurons. Uusisaari and De Schutter (2011) characterised six neural subtypes in the cerebellar nuclei: the smallest glutamatergic and glycinergic/ GABAergic interneurons, the intermediate to large glycinergic (two types) and GABAergic projection neurons and the largest glutamatergic projection neurons. In this study ~43% of small somata were β_1 IR whilst between 75%-85% of the other three size ranges were β_1 IR, indicating that the β_1-adrenoceptor is expressed by a restricted set of small interneurons, whilst a large number of all four of the intermediate to large projection neurons express the β_1-adrenoceptor. This observation suggests that β_1-adrenoceptor mediated activation may have a stronger influence over signalling in extra-nuclear regions (such as the olive and extra-cerebellar motor structures) than within the nuclei. Uusisaari and De Schutter (2011) indicate that there are two sub-classes of small interneuron in the cerebellar nuclei; a mixed inhibitory GABAergic/glycinergic class and putative glutamatergic class, suggesting the possibility that β_1-adrenoceptor expression is specific to one of these sub-classes. In contrast, the β_2-adrenoceptor is expressed in high proportions of cells in all size categories indicating β_2-adrenoceptor mediated signalling may have a more general role in modulating activity in the cerebellar nuclei.

2.4.5 Cerebellar nuclei cell size categorization: methodological limitations

Our categorization by somata size of the neuron type limits the conclusions to be drawn about β-adrenoceptors subtype expression. Currently the best method unambiguously to categorise neural types in the cerebellar nuclei is to identify the neurotransmitter they release (Uusisaari and De Schutter, 2011). However, it is our experience that in PFA fixed sections, the immunohistochemical markers used to distinguish cell neurotransmitter identity (e.g. anti-GAD67 for GABAergic neurons or

vGluT1/ vGluT2 for glutamatergic neurons) do not label cell somata but favour the GABAergic or glutamatergic terminals. At present, therefore, these methodologies could probably not be combined.

There were some additional drawbacks with the somata size approach to establishing nuclear neuron identities. First, from visual inspection, it could be seen that the protocol used to isolate the cell somata signal from dendritic or axonal processes was not always successful. For example if two somata were closely apposed the erosion/ dilation process could fail to separate them, and if many labelled processes closely surrounded the cell somata the erosion/ dilation could also fail to isolate the somata. Because of the high density of overlapping somata and processes in the cerebellar nuclei an alternative measurement strategy will be needed.

Unambiguous identification of cell type distribution in the nuclei might be achieved with other immunohistochemical markers for neuronal somata or a transgenic approach in which a reporter gene such as EGFP is selectively expressed in glutamatergic, GABAergic or glycinergic cells which can then be combined with IHC labelling of β-adrenoceptors. Nonetheless, the central aim of this study was to determine the expression of the β-adrenoceptors in the different cerebellar nuclei in order to aid interpretation of previous behavioural studies (Paredes et al, 2009) and this much is now established

2.4.6 Conclusion

The observations suggest that the noradrenergic system makes a significant contribution to network activity in the cerebellar cortex and the cerebellar nuclei and learning processes. Of special importance is the observation that the β_1 and β_2-adrenoceptors have entirely distinct cellular distributions in the cerebellum and that this will have important implications for understanding the behavioural outcomes of β-adrenoceptor manipulation in learning tasks.

Chapter 3: Distribution of monoaminergic fibres in the cerebellar cortex

3.1 Introduction

3.1.1 Characteristics of monoaminergic afferents in the brain

Monoaminergic fibres in the brain are often referred to as 'beaded' fibres (Ito, 2006) because of the characteristic neurotransmitter release sites, the varicosities, along their length. On the basis of the widespread distribution of monoaminergic afferents throughout the CNS and the failure to observe synaptic specialisations on their varicosities, early accounts of the monoaminergic afferents assumed they signalled by volume transmission and modulated targets distant from their varicosities (estimates in the tens of micrometres. Beaudet and Descarries, 1978; Agnati et al, 1995; Descarries and Mechawar, 2000). However, later evidence has demonstrated that large proportions of varicosities on monoaminergic afferents in many terminal regions do form synaptic junctions (up to 95%. Descarries and Mechawar, 2000 Papadopoulos et al, 1989; Papadopoulos and Parnavelas, 1990; Dinopoulos et al, 1995). A recent study of noradrenaline afferents in the basolateral amygdala found approximately 50% of varicosities have conventional synaptic specialisations (Zhang et al, 2013). Thus, monoaminergic afferents appear to be capable of both volume and synaptic transmission and there are regional differences in the extent to which each is favoured. Additionally, modern estimates of the effective distance of influence for volume transmission are significantly smaller than previously believed (Sykova and Nicholson, 2008; Taber and Hurley, 2014; Vargova and Sykova, 2014). Consequently, the distribution of noradrenergic and serotonergic afferents within the cerebellum is likely to closely match the area of influence of released NA and 5-HT.

3.1.2 What is known of noradrenergic afferents in the cerebellum?

The presence of noradrenergic afferents in the cerebellum was first revealed using histofluorescence (Falck-Hillarp method) by Hökfelt and Fuxe (1969) who observed a plexus of fine varicose fibres in all three layers and in all regions of the rat cerebellar

cortex and the cerebellar nuclei. Using a similar histofluorescence technique Bloom et al (1971) reported the specific pattern of innervation for noradrenergic (NA) fibres in the three layers of the cerebellar cortex. They observed the densest innervation of fibres in the PCL and in the upper third, and lower third, of the GCL and ML, respectively. The majority of the fibres in the GCL were observed to be oriented radially, extending towards the overlying PCL and ML, then at the upper third of the GCL the fibres begin to run parallel to the pial surface clustering close to the basal pole of the PCs. Many fibres within the PCL exhibited 'chains' of varicosities surrounding the PC somata. A similar pattern of innervation with a high density of fibres and varicosities clustered in the PCL have been reported using a range of methodologies in rat (Landis and Bloom, 1975; Swanson and Hartman. 1975; Yamamoto et al, 1977; Kimoto et al, 1981; Verney et al, 1982) and mouse cerebellum (Landis et al, 1975; Felten et al, 1986). There is a sparse plexus of NA fibres in all compartments of the cerebellar nuclei in mouse (Landis et al 1975; Felten et al 1986) and rat (Hökfelt and Fuxe, 1969; Swanson and Hartman, 1975).

3.1.3 What is known of serotonergic afferents in the cerebellum?

The first demonstration of putative 5-HT afferent fibres in the cerebellum came from a study by Hökfelt and Fuxe (1969). Using the Falck-Hillarp method after incubation of sections with 6-hydroxytryptamine, an indoleaminergic molecule that the same authors had previously shown was a critical step for visualising 5-HT afferents, a different fibre type was seen in addition to noradrenergic afferents: a very fine varicose fibre type in the ML that runs parallel to the long axis of the folium, which the authors concluded were serotonergic afferents. Chan-Palay (1975) used intraventricular infusions of tritiated 5-HT to trace serotonergic afferents in rats and rhesus monkeys. They observed a diffuse plexus of 5-HT afferent fibres in the GCL and ML including parallel fibre-like afferents in the ML and a plexus of fibres throughout all of the cerebellar nuclei. Similar cortical and nuclear distributions were seen using the more sensitive method of 5-HT IHC (Takeuchi et al 1982). They observed a low density of short fibres with no specific orientation in the GCL and very little evidence of release sites in the GCL or PCL, instead a high density of varicosities was seen on 5-HT afferents in the ML.

3.1.4 Glutamatergic afferents and the functional organisation of the cerebellar cortex

The organisation of the cerebellar cortical circuitry is precise and highly stereotyped (Eccles et al, 1967), a feature that indicates it may implement a universal computational procedure (Marr, 1968; Albus, 1971; Gilbert, 1975; Ito, 1982; Yeo and Hesslow, 1998) with the functional properties of each region depending on its afferent and efferent connections (Porrill et al, 2013). The most numerous cerebellar afferents are the mossy fibres that transmit information of various modalities from a range of precerebellar sources. They provide the Purkinje cell with this diverse input via granule cell parallel fibres that project for several millimetres in the long axis of the folium targeting many hundreds of Purkinje cells along their path. In contrast, a second cerebellar afferent, the climbing fibre branches to innervate only a few Purkinje cells and each Purkinje cell receives only a single climbing fibre branch. Climbing fibres originating from different olivary regions innervate restricted strips of the cortex that run transverse to the long axis of the folium and so divide the cortex into functional zones. These longitudinal zones can be further subdivided into 'microzones' that receive climbing fibre input from subsets of olivary neurons that share a specific receptive field. Each microzone controls a specific muscle or muscle group, via output to the cerebellar nuclei, with functionality related to its olivary receptive field.

Thanks in part to this understanding of the anatomical distribution of MFs and CFs within the cortical circuitry a comprehensive account of the contribution of each afferent to cerebellar learning has been established. Because of their specific anatomical and physiological characteristics, it has been proposed that mossy/parallel fibres provide information about context and climbing fibres provide a teaching signal that alters PC activity. Coincident mossy/parallel fibre and climbing fibre signalling drives cortical plasticity that underpins motor learning, traditionally claimed to be parallel fibre to Purkinje cell synaptic LTD (Ito and Kano, 1982; Ito, 1989). In contrast to the comprehensive accounts of the distribution of MFs, PFs and CFs relatively little is known concerning the distribution of 5-HT and NA afferents in relation to the general structure and zonal organisation of the cerebellum. However a small number of earlier reports have provided some insight into the anatomical organisation of 5-HT and NA afferents. Verney et al (1982) reported that NA fibres in the ML follow a parasagittal path parallel to the PC dendritic arbor in a manner similar to the orientation of CFs. In contrast Chan-Palay (1975) and Takeuchi et al (1982) observed 5-HT fibres in the ML running perpendicular to the PC dendritic arbor, similarly to PFs. However, as yet, no

systematic analysis of the distribution monoaminergic fibres in relation to the zonal organisation of the cerebellar cortex has been undertaken.

3.1.5 Theoretical accounts of monoaminergic afferents to the cerebellar cortex and their functional organisation

A model from Schweighofer et al (2004) suggests a functional framework for understanding the contribution of monoaminergic afferents to cerebellar information processing, including cerebellar learning. The model is of interest here because it introduces testable predictions about the necessary cortical distribution of 5-HT and NA afferents. Schweighofer et al (2004) suggest that 5-HT acts as a modular 'responsibility' signal, simultaneously influencing spatially separated but functionally connected microzones modulating their output to premotor targets and orchestrating shared plasticity. Further, they claim that the NA afferent system broadcasts a gating signal under conditions in which learning in the cerebellum would be appropriate, thus permitting the occurrence several forms of synaptic plasticity under appropriate circumstances and minimising the occurrence of plasticity under conditions where learning is not required. Furthermore, they propose that NA is a diffuse signal simultaneously released across all microzones and local coincident CF and MF activity limits which microzones are receptive to the signal.

Thus, the model by Schweighofer at el (2004) makes some testable anatomical predictions. 5-HT afferents would need to collateralize before reaching the cerebellar cortex in order to target spatially separate but functionally related microzones and their termination in the cerebellar cortex would be restricted to sets of functionally related microzones. In contrast, noradrenaline afferents would not require spatial restriction in their cortical terminations and we might expect individual NA fibres to cover large regions of the cerebellar cortex. As reviewed above there is some preliminary evidence that 5-HT afferents travel across the cerebellar cortex in the ML rather like parallel fibres. If these trajectories are extensive, they would be incompatible with the predictions of Schweighofer et al (2004) because they would probably influence any and all microzones that they traverse, rather than a selection of functionally related ones. However, there has been no systematic analysis of the spatial organisation of the 5-HT and NA afferents in the cerebellum sufficient to define their relationships with zonal and microzonal organisation. The studies here provide those analyses and allow

evaluation of the Schweighofer et al (2004) model and provide the foundations for others.

3.1.6 Experimental summary

Antibodies generated to the noradrenaline and 5-HT transporters (NET and SERT) have previously been used effectively to label noradrenergic and serotonergic afferents in other regions of the brain (Qian et al, 1995; Schroeter et al, 2000; Nielsen et al, 2006; Zhang et al, 2013). We used NET and SERT antibodies to label noradrenergic and serotonergic afferents and systematically examined their cortical distribution and orientation. In particular we focused on their orientation in relation to the parasagittal zonal architecture of the cerebellar cortex.

3.2 Methods

3.2.1 Animals and Material preparation

Sixteen adult female Sprague Dawley rats (weight: approximately 250g) were used. All procedures were approved by the local ethical review panel of University College London and were in accordance with the UK Home Office Animals (Scientific Procedures) Act under the provision of licence 70/23405. Perfusion and postfixation procedures were as those in Chapter 2 (see section 2.2.1). Brains were cut in serial 40µm or 200µm coronal sections or serial 40µm parasagittal sections depending on the experiment.

3.2.2 Immunohistochemistry

Prior to beginning conventional immunohistochemistry procedures, 200µm sections underwent treatment to improve antibody penetration in thick tissue. First, the sections were incubated for 15 minutes in a solution of 20% sucrose in distilled H_20 for cryoprotection. Sections were stored individually in Eppendorf tubes containing the same 20% sucrose solution throughout three freeze-thaw cycles of 15 minutes at -50°C and 45 minutes at ambient temperature. Sections were then washed three times for five minutes in 0.1M TBS before undergoing mild proteinase K digestion (10mg/ml) for five minutes. Finally sections were washed three times for 20 minutes in 0.1M TBS to fully remove any residual proteinase K before immunohistochemistry procedures were started.

The blocking solution was normal goat serum (2.5% w/v), Tween 100 (0.05% w/v), bovine serum albumin (1%) and Triton X (0.8% w/v for 200 µm sections or 0.2% w/v for 40 µm sections). All antibodies were diluted in this blocking solution at concentrations shown in Table 3.1 in the 0.2% w/v Triton X blocking solution. Wash steps for 40 µm sections involved three 10 minute incubations in 0.1M TBS. Wash steps for 200 µm sections involved three 40 minute incubations in 0.1M TBS. Timings for 40 µm and 200 µm sections were different throughout so procedures will be described separately.

40 µm sections:

Sections were incubated in blocking solution for one hour at room temperature. Sections were then incubated for 6 hours at room temperature plus overnight at 4°C then washed. Secondary antibodies were applied for 2 hours at room temperature, secondary antibodies were anti-mouse Biotin-XX (NET) and anti-rabbit Biotin-XX (SERT) and anti-mouse or anti-rabbit Alexa-Fluor 594 for double labelling experiments (see table 3.1 for a list of antibodies used). Sections were then washed and incubated in Alexa-Fluor 488 conjugated streptavidin for one hour at room temperature before a final set of washes. Sections were then mounted onto microscope slides in VECTASHIELD mounting media (Vector Laboratories) and coverslipped. Coverslips were sealed to the slides and these were stored in aluminium foil to prevent light entry and at 4°C to preserve tissue and fluorescence quality. Sections were examined and imaged with fluorescence or confocal and multiphoton microscopy within seven days in most cases and within two weeks in all cases.

200 µm sections:

Sections were incubated in blocking solution overnight at room temperature. Sections were then incubated in primary antibody for 24 hours at room temperature then washed. Secondary antibodies were applied for 6 hours at room temperature plus overnight incubation at 4°C. Sections were then washed and incubated in Alexa-Fluor 488 conjugated streptavidin for four hours at room temperature before a final set of washes. Slide mounting was the same as above.

3.2.3 Fluorescence Microscopy

All initial observations of the labelled sections were with conventional, fluorescence microscopy using a Zeiss Axioscope with filters optimised to view FITC and Rhodamine fluorophores. Target regions for examination with confocal and multi-photon microscopy were identified.

Antigen	Structures labelled	Ab type	Dilution	Final dilution	Secondary/ tertiary detection	Company/ Catalogue #
Noradrenaline transporter *(NET)*	Noradrenergic fibres	Mouse Mono.	1:200; 500; 1000	1:1000	Goat biotinylated anti-mouse; AF 488 conjugated streptavidin	MAb Technologies/ NET05-2
Serotonin reuptake transporter *(SERT)*	Serotonergic fibres	Rabbit Poly.	1:250; 500; 1000	1:1000	Goat biotinylated anti-rabbit; AF 488 conjugated streptavidin	Millipore/ AB9726
Calbindin (CALB1)	PC dendritic arbors	Mouse mono.	1:500; 1000; 1500	1:1500	Goat anti-mouse AF 488 or 594	Millipore/ AB1778
β_1-adrenoceptor (B1)	PC dendritic arbors	Rabbit poly.	1:250; 500; 1000	1:500	Goat biotinylated anti-mouse; Alexa-Fluor (AF) 594 conjugated streptavidin	SCBT/ sc-568
Aldolase C	Parasagittal stripes of PCs	Rabbit Poly.	1:500; 1000; 1500	1:1000	Goat anti-rabbit AF 594	Gift. Professor Izumi Sugihara, Tokyo University
Zebrin II (ALDOC)	Parasagittal stripes of PCs	Mouse mono.	1:500; 1000; 1500	1:1000	Goat anti-mouse AF 594	Gift. Professor Richard Hawkes, Calgary University
Goat anti-rabbit biotin-XX		Goat poly.		1:200		Invitrogen/ B2770
Goat anti-mouse biotin-XX		Goat poly.		1:200		Invitrogen/ B2763
Alexa-fluor 488 conjugated streptavidin				1:1000		Invitrogen/ S11223
Goat anti-mouse Alexa-fluor 594		Goat poly.		1:400		Invitrogen/ A11005
Goat anti-rabbit Alexa-fluor 594		Goat poly.		1:400		Invitrogen/ A11037

Table 3.1. Summary of primary antibodies and secondary antibody used for their detection. Ab: antibody, mono: monoclonal, poly: polyclonal, AF: Alexa-Fluor

3.2.4 Confocal and Multi-photon Microscopy

Digital micrographs of 40 µm sections were collected using an Olympus FV-1000 TIRF confocal laser scanning microscope with appropriate excitation lasers (488nm and 559nm for Alexa-Fluor 488 or 594 fluorochromes). Digital micrographs of 200 µm sections were collected using a Leica TCS SP8 MP (multi-photon) microscope. Use of a multiphoton microscope allowed for deeper laser excitation penetration in thick sections. Lobules of interest were first located in the vermis using epifluorescence

mode before multi-micrograph montages that encompassed a large region of the chosen lobule/ folium were collected using the Olympus or Leica tracking stages. The parasagittal sections used for this experiment were from midline vermis and coordinates were specified to encompass an entire lobule/folium of interest. Montages from coronal sections were specified to encompass the midline region of the lobule of interest. The general procedure for collecting multi-micrograph montages using the Leica microscope and tracking stage was essentially identical to that using the Olympus microscope (see section 2.2.3).

The coronal and parasagittal planes are either parallel to, or perpendicular to, respectively, the long axis of the folium strictly only in the midline vermis. For this reason, our analyses focused on this midline vermis region in order to be certain of the orientation of the measured fibres in relation to the parasagittally organised zones and microzones. Images obtained using the Olympus confocal microscope were collected using a x20 objective and those obtained using the Leica multiphoton microscope were collected using a x25 water-immersion objective with coverslip correction. Each individual micrograph of the montage was a z-stack series with images collected at 0.8 µm steps through the entire depth of the section.

3.2.5 Image analysis and data analysis

All confocal digital micrographs were analysed and prepared for presentation using Fiji software (Schindelin et al., 2012). First, z-stack digital micrographs were opened using Fiji and histogram equalization optimised image contrast. In Fiji, a line tracing the approximate course of the PCL was overlaid on to the image, transects were drawn at every 300 µm perpendicular to the PCL through the molecular layer and the lengths of all fibres crossing these transects were measured using the Simple Neurite Tracer plugin (Longair et al., 2011). Raw fibre measurements were exported as a .csv file and collected in Microsoft Excel. In this experiment we examined the length of noradrenergic and serotonergic fibres in the rostrocaudal or mediolateral plane in the ML. Using the total measured length of a fibre, particularly if the fibre were either radially or obliquely oriented or with a tortuous path, would overestimate the distance it covers in the mediolateral or rostrocaudal plane. To avoid bias in estimates of SERT+ and NET+ fibre coverage in these planes, we also measured the distance covered by each measured fibre in a line at constant distance from the PCL in either the medial-lateral or rostral-caudal plane. All fibre length measurements presented in the results

section are ‘PCL fibre lengths’ not total fibre lengths. Micrographs chosen for presentation in Figures were condensed to a single image using the ImageJ maximum intensity projection command and scale bars were added.

Descriptive statistics were calculated in Microsoft Excel for fibre length measurements for SERT/40 µm/coronal, SERT/40 µm/parasagittal, SERT/200 µm/coronal, NET/40 µm/coronal, NET/40 µm/parasagittal and NET/200 µm/coronal. Statistical tests were performed using SPSS 19. Because of the number of cases per group (40 µm = 6 cases per group. 200 µm = 5 cases per group) non-parametric statistical tests were used.

3.3 Results

The laminar distribution and topographical organisation of noradrenergic and serotonergic fibres in the cerebellar cortex of adult rats was examined with immunohistochemistry. The topographical organisation of glutamatergic climbing fibre afferents and their targets define parasagittal zones. Noradrenergic and serotonergic fibre orientation was examined at, or close to, the midline in coronal and parasagittal vermis section where sections run transverse or parallel (respectively) with the long axis of these parasagittal zones and microzones.

3.3.1 NET and SERT immunolabelling revealed noradrenergic and serotonergic fibre distributions widely through the cerebellum

The presence of NET immunoreactivity (NET+) was evidence for noradrenergic fibres and the presence of SERT immunoreactivity (SERT+) was evidence for serotonergic fibres. NET+ and SERT+ fibres were seen in all three layers of the cerebellar cortex when examined in 40 µm coronal sections (Fig. 3.1A, B, Y and Z). Fibres were present across the entire cerebellar cortex regardless of region: vermis, paravermis and hemispheral (including paraflocculus/ flocculus) regions were innervated by NET+ and SERT+ fibres. No NET IR was seen on cell somata or dendritic processes but some low levels of SERT IR was seen on putative PCs in most sections. This may be due to postsynaptic expression of SERTs by PCs similar to postsynaptic expression seen in non-serotonergic neurons in other regions of the CNS.

3.3.2 NET immunolabelling in coronal sections reveals that molecular layer noradrenergic fibres have restricted distributions in the medial-lateral plane

The vast majority of NET+ fibres in the molecular layer are short or punctate (Fig. 3.1Y white arrowheads) with only a small number of longer fibres present. Most NET+ fibres measured in the ML in coronal vermis are short, many extending as little as 20 µm in the medial-lateral plane (~35%). In sections double labelled for aldolase C (Zebrin II) a marker for parasagittally oriented subsets or ‘stripes’ of PCs the majority of individual NET+ fibres seen are restricted to a single aldolase C+ or aldolase C- stripe, with only a small number spanning the boundary between aldolase C+ and – stripes (Fig. 3.1A and Y).

The longer ML fibres fall into two broad categories: radially oriented fibres (Fig. 3.1Y black arrowheads) and horizontal fibres, running approximately parallel to the PCL (Fig. 3.1Y arrows). A small proportion of the horizontal fibres measured in vermis extend for 150-300 μm parallel to the PCL (~5% of total fibres measured) and a very small number extend >300 μm (<1%). In the GCL, NET+ fibres appear as short puncta with no obvious orientation or as radially oriented fibres extending towards the PCL and ML (Fig. 3.1A and Y). In the PCL NET+ fibres also appear short or punctate but in a slightly higher density than in the GCL and, in particular, high densities of short fibres are seen around putative PC somata with some patches of the PCL appearing to have higher densities of NET+ fibres surrounding PCs then other patches (Fig. 3.1A black arrows).

3.3.3 SERT immunolabelling in coronal sections reveals extensive medial-lateral 5-HT fibre projections in the molecular layer

In the molecular layer, the orientation of SERT+ fibres are strikingly different to NET+ fibres. The majority of SERT+ fibres are long and horizontal, running extensively in the medial-lateral plane parallel to the PCL, often for several hundred micrometres (Fig. 3.1B and Fig. 3.1Z white arrowheads). Of the SERT+ fibres measured in the coronal plane ~50% were between 150-300 μm and 10% were >300 μm in their medial-lateral extent. Double labelling sections with Zebrin II to reveal parasagittal stripes revealed many SERT+ fibres crossing at least one boundary between an adjacent Zebrin II+ and Zebrin II- stripe with many seen crossing multiple stripes (Fig. 3.1B and Z). This highly characteristic pattern strongly resembles the trajectory of parallel fibres. There were also a small number of radially oriented SERT+ fibres and very few short or punctate SERT+ fibres in the ML. The radially oriented fibres are consistent with fibre trajectories through the lower parts of the ML before the fibre branches or bifurcates to produce the parallel fibre-like patterns within the ML (Fig. 3.1Z black arrows). The small number of short and punctate SERT+ fibres is consistent with the suggestion that only a very small number of SERT+ fibres have other trajectories.

In contrast to the pattern seen in the ML in the GCL in coronal sections SERT+ fibres are mostly of the short variety with no specific orientation, some longer fibres can be seen and these are usually oriented radially and are probably segments of fibres extending towards terminal fields in the ML (as suggested by Takeuchi et al, 1982). A high density of short SERT+ fibres were seen in the PCL. The most common type of

SERT+ fibre seen is a short or punctate fibre, as in the GCL, but with slightly higher densities, particularly around putative PC somata.

3.3.4 Summary of distribution patterns of noradrenergic and serotonergic fibres in coronal sections.

The patterns seen in sections from coronal vermis indicate that NET+ fibres are highly restricted in their medial-lateral orientation whilst SERT+ fibres cross large regions of the ML in the medial-lateral plane. SERT+ fibre orientation is particularly reminiscent of PFs that extend for long distances in the long axis of the folium, crossing multiple parasagittally oriented microzones with no significant deviation in the rostral-caudal plane. In contrast, the high numbers of short or punctate NET+ fibres and very small number of horizontally oriented NET+ fibres in coronal sections of the vermis suggests noradrenaline afferents may be oriented in the parasagittal plane, aligned with zones or microzones. Thus, coronal sections are more likely to transect them, leading to the punctate appearance. An alternative possibility is that NET+ fibres have no extended trajectories in the rostral-caudal or medial-lateral planes but are mostly radially oriented, entering the ML and extending radially towards the pial surface with only small branches of random orientation.

The results from analysis of coronal sections suggest that serotonergic afferents in the ML have a strong anisotropy and follow trajectories similar to those of PFs along the medial-lateral plane. They further suggest that noradrenergic afferents may also have a strong anisotropy and follow trajectories in the rostral-caudal plane. To test these possibilities, we next examined both types of monoaminergic afferent in parasagittal sections and focused our investigations on the midline vermis, where the planes of section are orthogonal to the long axes of the folia (see Methods section for an explanation).

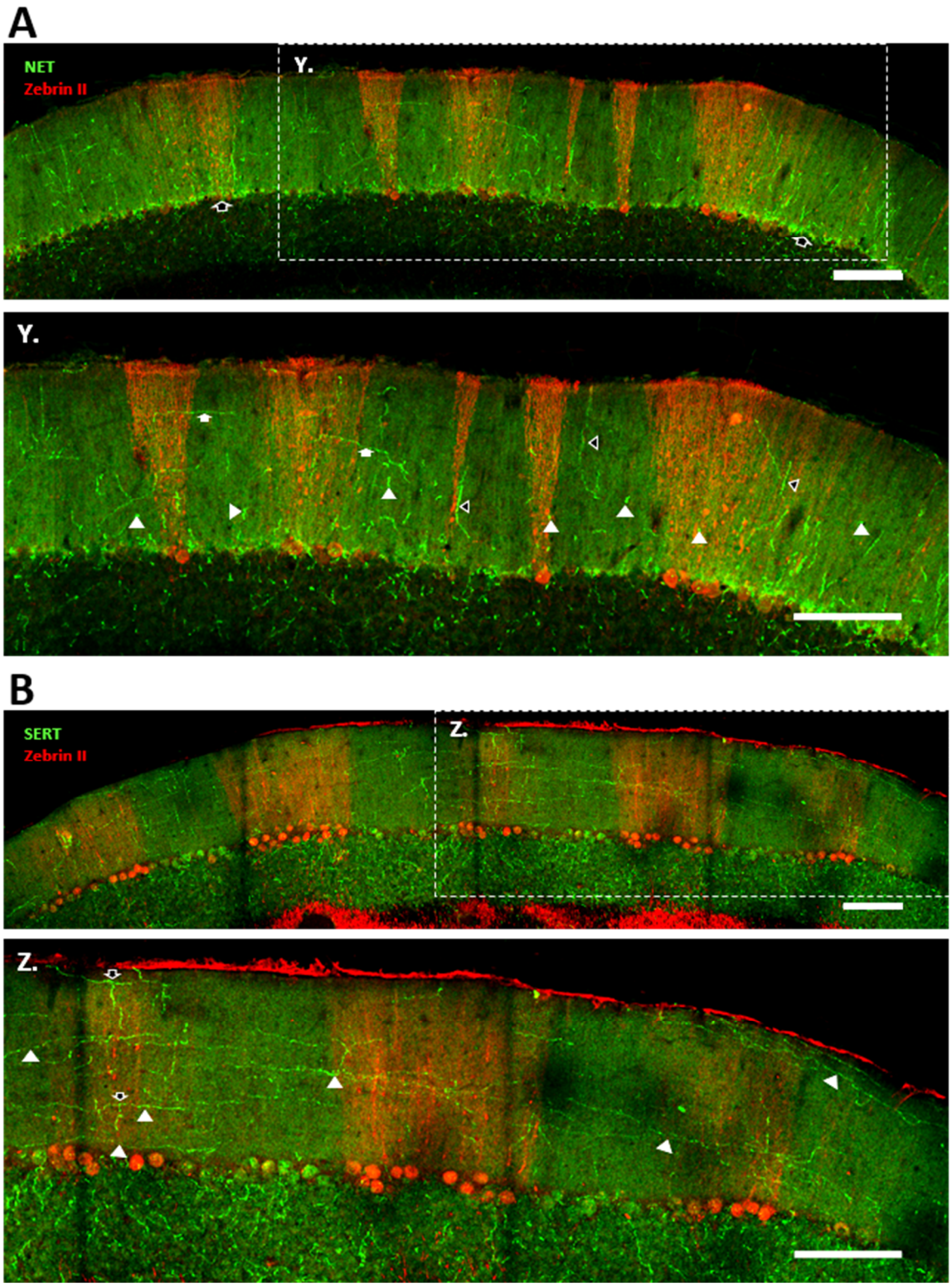


Fig 3.1 – Legend overleaf.

Fig. 3.1: NET and SERT labelling in coronal sections reveal contrasting distributions of noradrenergic and serotonergic fibres in the molecular layer. A) NET+ fibres distribution is restricted in the medial-lateral plane with many fibres appearing as small puncta in coronal sections (A and Y: white arrowheads). Some long radial fibres can be seen (A and Y: black arowheads), but very few long transverse fibres (>100μm) are seen (A and Y: arrows) the majority are restricted to a single aldolase C + or - band. Some patches of high densities of NET+ are seen in the PCL (black arrows). **B)** SERT+ fibres extend for long distances in the medial-lateral plane (B and Z: white arrowheads). Many fibres pass through multiple Zebrin II+ and - bands. Sections double labelled with aldolase c (A)/ Zebrin II (B). Scale bars: 150μm.

3.3.5 Molecular layer NET+ fibre distribution in parasagittal sections is strongly anisotropic, with multiple rostral-caudal trajectories. Comparisons with coronal sections confirm the anisotropy

Patterns of NET+ fibres revealed in parasagittal sections were strikingly different from those revealed in coronal sections in the molecular layer (Fig. 3.2A, B). The clearest difference is the much higher prevalence of long fibres running parallel to the PCL (150-300 µm: ~50%; >300 µm: ~15%). This difference suggests a strong anisotropy in the orientation of NET+ fibres. They run predominantly in the rostral-caudal plane. When the length of ML NET+ fibres in parasagittal sections (n=6) and coronal sections (n=6, descriptive statistics in table 3.2) are compared the mean NET+ ML fibre lengths are much longer in parasagittal sections then in coronal sections (Fig. 3.3) and this difference is significant (Mann-Whitney test $p<0.01$). The strong inference to be drawn here is that individual noradrenergic afferents have a restricted distribution perhaps even within one microzone. Some short or punctate and radial NET+ fibres can still be observed in the ML but far fewer than in coronal sections.

In addition to differences in the ML differences in NET fibre orientation between parasagittal and coronal sections were also observed in the GCL. A higher number of long fibres were seen in the GCL in parasagittal sections than in coronal sections (Fig. 3.2A, B). In particular, in the bottom and top third of the GCL and in the underlying white matter many fibres travelled extensively in the rostral-caudal plane (Fig. 3.2B red arrowheads). This observation suggests that NET+ fibres project for long distances in the rostral-caudal plane in the white matter and GCL before extending radially towards the PCL and ML. Alternatively, single fibres may project for long distances in a rostral-caudal direction giving off multiple collaterals to the overlying PCL and ML along their path. That this pattern in the GCL is not seen in midline coronal sections suggests that, after entering the cerebellum, NET+ fibres may travel strictly within restricted parasagittal compartments in the white matter and GCL. As in coronal sections (Fig. 3.1A black arrows), a high density of NET+ fibres can be seen in the PCL, where small regions of high density of NET+ fibre can be distinguished from areas of lower density (Fig. 3.2B black arrows). This pattern appears to part of the weakly parasagittal organisation of these NET+ fibre densities seen more clearly in coronal sections – as described above. Together, these observations suggest patchy concentrations of NET+ fibres in the PCL, rather than full rostral-caudal or medial-lateral bands.

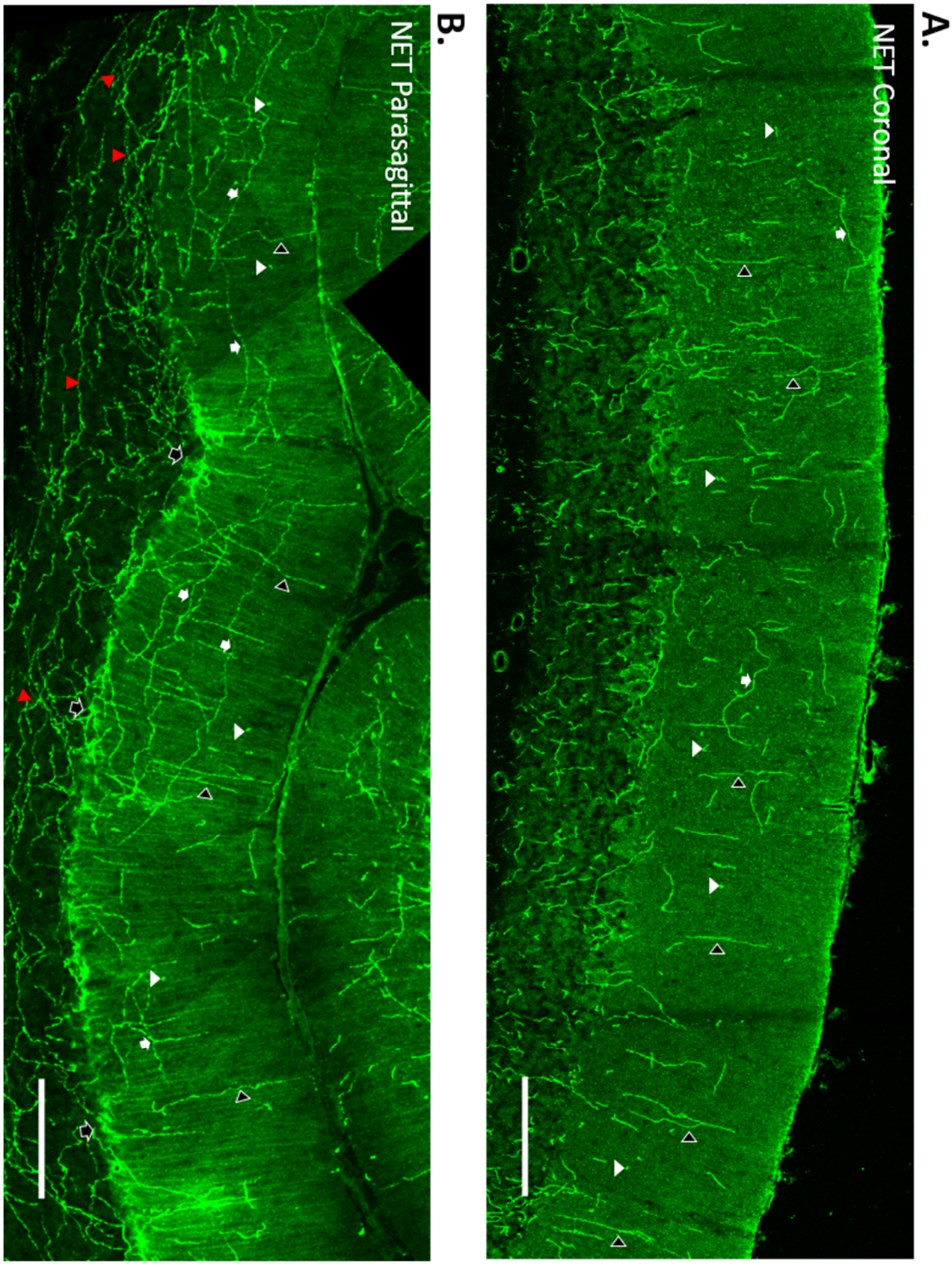


Fig. 3.2 – Legend overleaf.

Fig. 3.2: Comparison of noradrenergic fibre distribution in coronal and parasagittal sections reveals strong anisotropy in the molecular layer. A) In coronal sections NET+ fibres are restricted in the medial-lateral plane with many small NET+ puncta (A: white arrowheads), some radial fibres can be seen (A: black arrowheads) and only very few long transverse fibres (A: white arrows) (see also Fig. 3.1). **B)** In parasagittal sections at the midline far fewer NET+ small puncta are seen (B: white arrowheads), approximately as many radial fibres can be seen (A: black arrowheads) but a much larger number of long fibres (up to 500µm in Fig. 3.2B) running parallel to the PCL are seen in the molecular layer (B: arrows). In parasagittal sections, but not coronal sections, high densities of NET+ fibres are seen in discrete regions of the PCL and adjacent ML and GCL (black arrows: B) and long rostral-caudal fibres can also be observed in the GCL and underlying white matter (red arrowheads) Scale bars: 150µm.

3.3.6 Molecular layer SERT+ fibre distribution in parasagittal sections is strongly anisotropic with multiple medial-lateral trajectories. Comparisons with coronal sections confirms the anisotropy

In the molecular layer there is a clear difference between SERT+ fibres seen in parasagittal and coronal sections (Fig. 3.4A, B). Those seen in coronal sections are predominantly long, running parallel to the PCL whereas those seen in parasagittal sections are almost universally short or punctate in appearance (<50 μm: ~70%). The difference in mean SERT+ fibre length for coronal and parasagittal sections is statistically significant (Mann-Whitney test $p<0.01$. n=6 for coronal and parasagittal sections. Fig. 3.3. Descriptive statistics in table 3.2). This difference indicates a strict anisotropy in the serotonergic afferent projection. Serotonergic fibres appear to distribute in a manner similar to PFs, targeting PCs and spanning multiple microzones and possibly crossing zonal boundaries. In parasagittal sections SERT+ fibres are predominantly short or punctate in the GCL and the PCL, as they are in coronal sections.

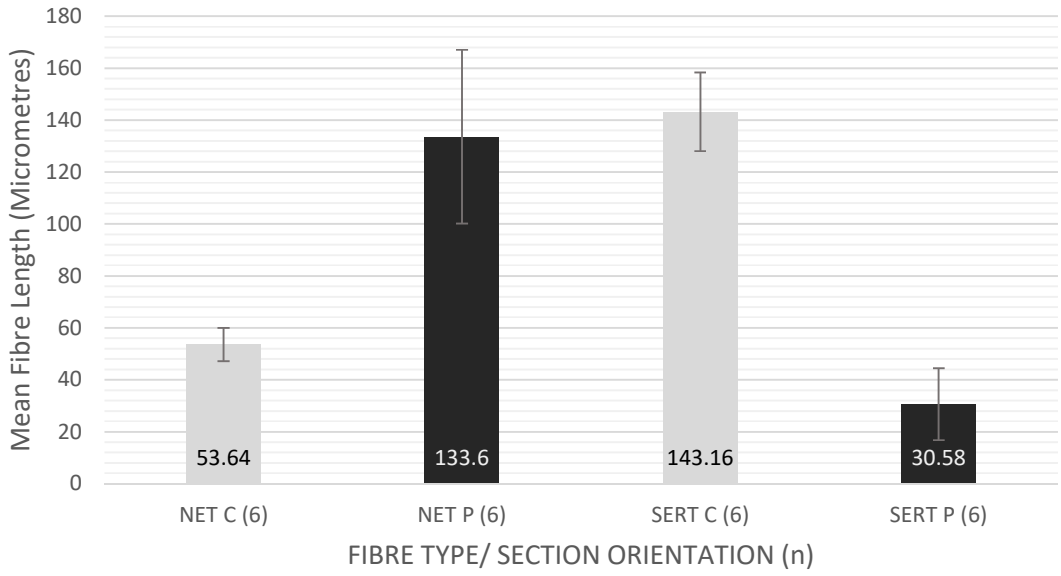


Fig. 3.3: Comparison of mean noradrenergic and serotonergic fibre lengths in coronal and parasagittal sections. Mean lengths presented in micrometres. Error bars: 95% confidence interval.

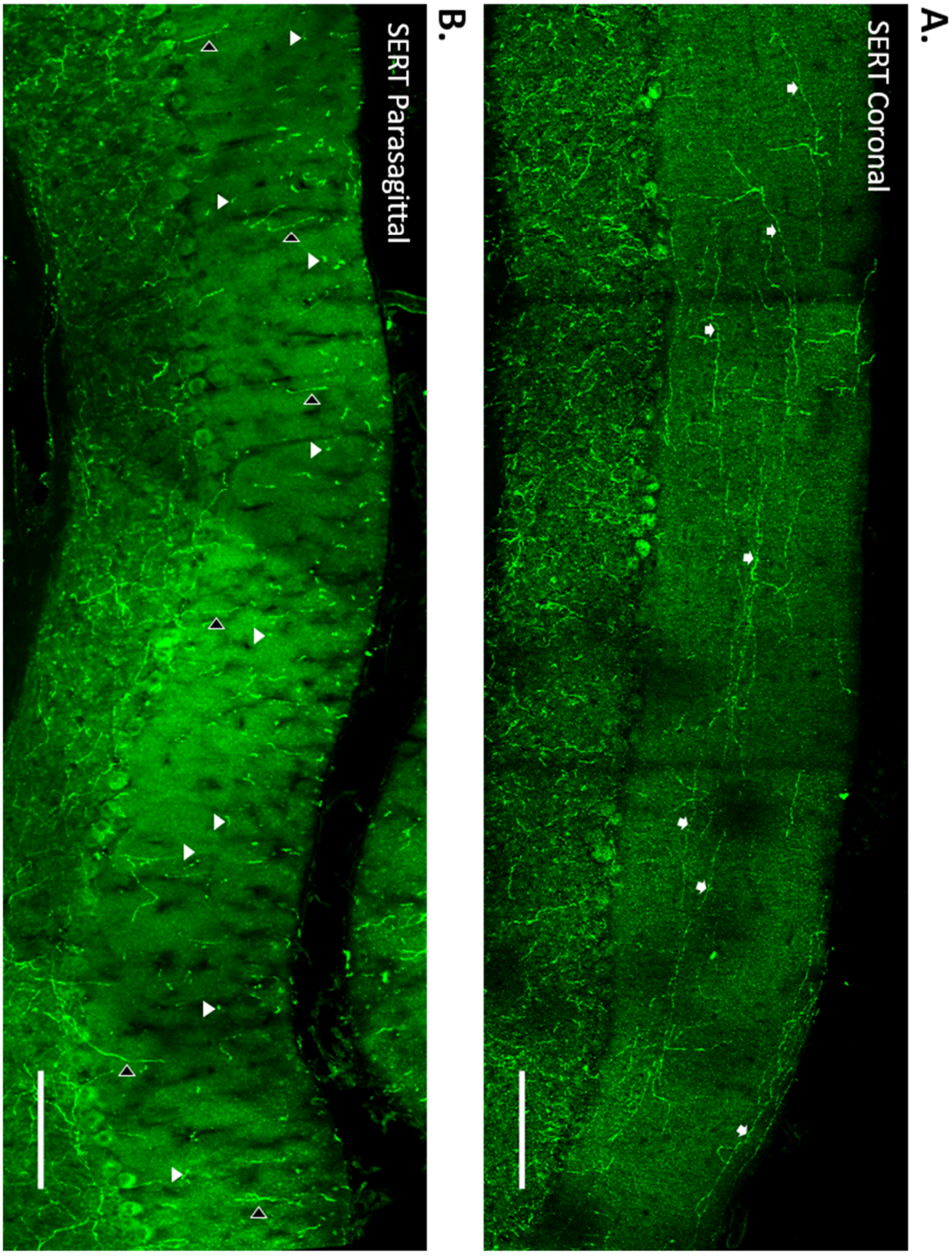


Fig. 3.4 – Legend overleaf.

Fig. 3.4: Comparison of serotonergic fibre distribution in coronal and parasagittal sections reveals a strong anisotropy in the molecular layer. A) The majority of SERT+ fibres seen in coronal sections extend parallel to the long axis of the folium in the molecular layer and extend for long distances (in Fig. 3.2A up to 500µm. A: arrows). **B)** In parasagittal sections the majority of SERT+ fibres are seen as small puncta and a smaller number are short and oriented radially (B: white arrowheads and black arrowheads respectively). There are no fibres extending parallel to the PCL for distances longer than 50µm. Scale bars: 150µm.

The differences in noradrenergic and serotonergic afferent distribution within the cerebellar cortical circuitry are very apparent when the patterns of NET+ and SERT+ fibre distribution are compared in parasagittal sections in which PC dendrites have been labelled by β_1-adrenoceptor or calbindin immunohistochemistry. The NET antibody used in this study was a mouse monoclonal type, therefore it could not be used in combination with the mouse monoclonal calbindin, so we used the rabbit polyclonal β_1-adrenoceptor antibody, which has been shown in Chapter 2 (Section 2.3.1) to label PC dendritic trees. The Purkinje cell dendritic arbor is strictly parasagittally oriented so parasagittal sections closer to the midline have broader and more fanlike PC dendritic arbors. In parasagittal sections where the PC dendritic arbor is most extensive, long NET+ fibres passing through multiple PC dendritic arbors are seen (Fig. 3.5A) whilst SERT+ fibres are often short or punctate and they traverse only a small region of the PC dendritic arbor before passing out of the section (Fig. 3.5B). This suggests that individual NET+ fibres may influence several PCs within the same parasagittaly organised microzone, potentially at multiple sites on an individual PC, whilst SERT+ fibres may influence many PCs oriented in the medial-lateral plane crossing multiple microzones and potentially crossing zonal boundaries.

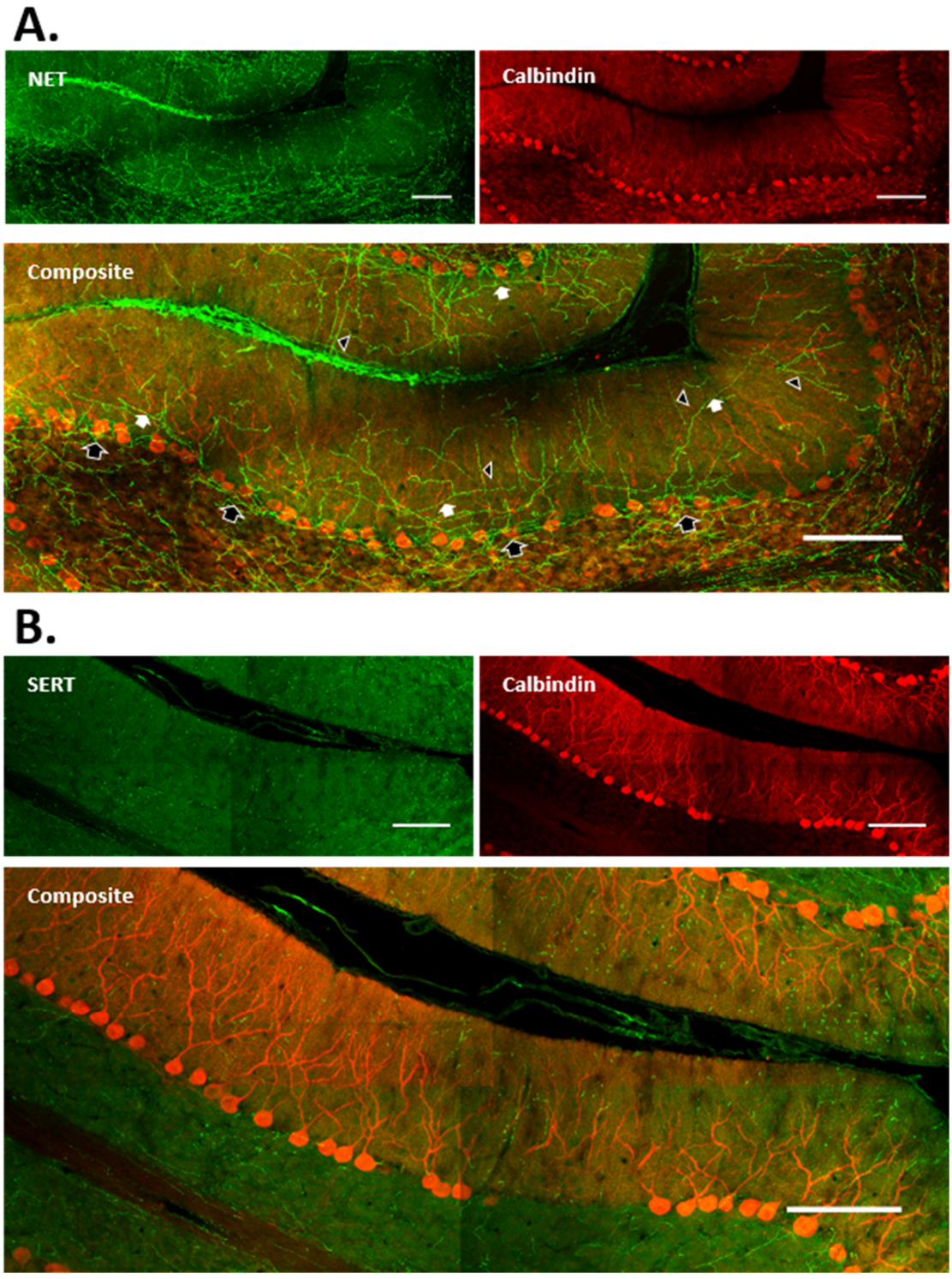


Fig. 3.5 – Legend overleaf.

Fig. 3.5: Double labelling for noradrenergic or serotonergic fibres and calbindin for Purkinje cells reveals the orientation of monoaminergic fibres relative to the PC dendritic arbor. A) In addition to radial fibres (A: black arrows) a large number of NET+ fibres are seen running parallel to the long axis of calbindin+ PC dendritic arbors (A: white arrows) indicating NET+ fibres run in line with the long axis of zones. In parasagittal NET+ sections, restricted regions of high densities of NET+ fibres are concentrated close to the PCL (A: black arrows). **B)** In contrast, SERT+ fibres are seen almost exclusively as small puncta in regions of parasagittal sections where the dendritic trees of calbindin+ PCs appear fanlike. Scale bars: 150µm.

3.3.7 Fibre measurements in 200 µm thick coronal sections confirm that molecular layer noradrenergic and serotonergic afferents have orthogonal orientations

Individual cerebellar cortical folia curve symmetrically on either side of the midline such that, in *coronal* sections, the strict relationship at the midline between the long axis of the folium and parasagittally organised zones is quickly lost with distance from the midline. If SERT+ fibres are very long in the medial-lateral plane and are aligned with the long axis of the folium, then estimates of SERT+ fibre length will relate to section thickness. As the long axis of the folium curves out of the section plane, thin sections (e.g. 40 µm) will underestimate length more than thick ones. NET+ fibres follow meandering paths and therefore are likely to leave the z-plane of section before their entire lengths can be measured. These features of SERT+ and NET+ fibres would lead to an underestimate of the medial-lateral extent of the fibres in the sections. In the case of SERT+ fibres it would restrict assessment as to whether individual SERT+ fibres cross multiple microzones or zones. In the case of NET+ fibres it may lead to an exaggeration of the degree of anisotropy of projection orientations. To address this, we cut 200 µm sections in the coronal plane to evaluate NET+ and SERT+ fibre lengths measured in these sections (n= 5 each for NET and SERT) and compare with NET+ and SERT+ fibres lengths measured in 40 µm coronal sections.

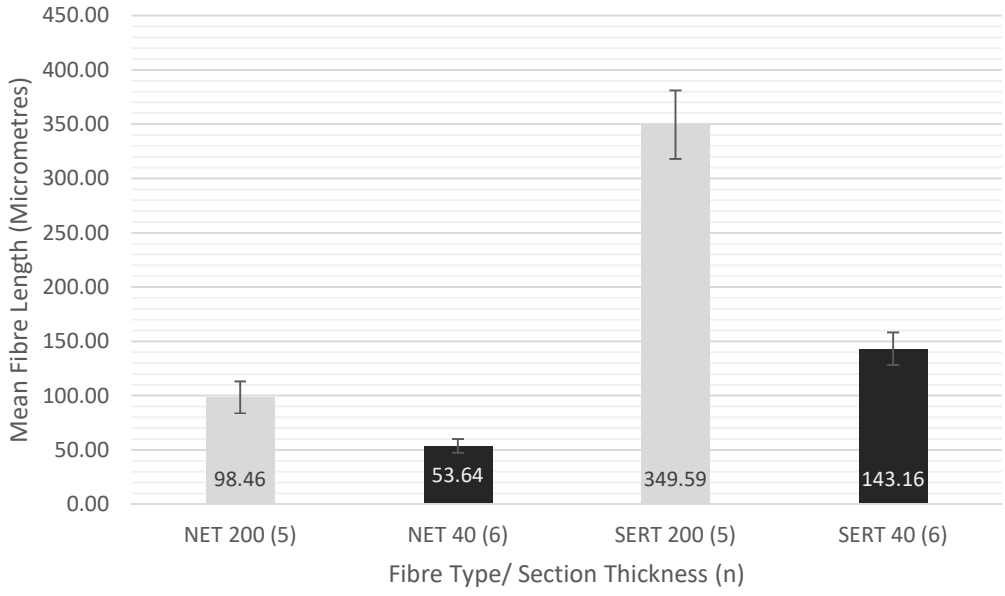


Fig. 3.6: Comparison of mean noradrenergic and serotonergic fibre lengths in coronal 200 µm and 40 µm coronal sections. Figures in brackets = n. Error bars: 95% confidence interval.

Fibre type/ Orientation	Section thickness	n.	Max	Mean	Median	S.D.	S.E.M.	C.I.
NET Coronal	40 µm	6	454.74	53.64	50.53	8.01	3.27	6.41
NET Parasagittal	40 µm	6	723.41	133.60	130.27	41.82	17.07	33.46
NET Coronal	200 µm	5	450.55	98.46	93.85	16.77	7.50	13.42
SERT Coronal	40 µm	6	757.18	143.16	152.54	18.92	7.72	15.14
SERT Parasagittal	40 µm	6	215.93	30.58	37.84	17.30	7.74	13.84
SERT Coronal	200 µm	5	898.19	349.59	331.30	35.97	16.09	31.53

Table 3.2: Descriptive statistics for noradrenergic and serotonergic fibre lengths measured in coronal (40 µm and 200 µm) and parasagittal (40 µm) sections. S.D. standard deviation; S.E.M standard error of the mean; C.I. 95% confidence interval

SERT+ fibre analysis

In 200 µm coronal sections (n=5. Fig. 3.7A and Z) the mean SERT+ fibre length is much higher than in 40 µm sections (n=6. Fig. 3.6 and table 3.1) and this difference is significant (Mann-Whitney test $p<0.01$). The maximum length of SERT+ fibres measured in 200 µm coronal sections was also much greater than that measured in 40 µm sections (898.19 µm and 757.18 µm respectively, table 3.1) and the proportion of >300 µm and 150-300 µm fibres was increased in 200 µm sections compared to 40 µm sections (200 µm sections: 150-300 µm: 30%; 300-600: 40%; >600: 10% in contrast to 40 µm sections: 150-300 µm: 50%; 300-600: 9%; >600: 1%). This suggests that despite the conspicuous medial-lateral nature of SERT+ fibres seen in the ML in 40 µm sections measurements from 40 µm sections may underestimate the serotonergic fibre field of influence in the medial-lateral plane. On average a zone in the rat is ~1-2 mm (for review see Apps and Hawkes, 2009) and the maximum length of measured SERT+ fibre was 0.9 mm in its medial-lateral extent. This indicates that if the maximum length of individual SERT+ fibres was captured here, fibres would certainly cross multiple microzones and could bridge zones. However, in 200 µm sections the long axis of the folium is also likely to curve out of the plane of section. Thus if SERT+ fibres do trace the folium, similarly to parallel fibres, then fibre lengths measured in 200 µm sections are still likely to be an underestimate of total length. The 200 µm section thickness was chosen for the present study for two reasons; first, because it allowed even antibody penetration throughout the tissue whilst allowing a short enough incubation time to minimise non-specific tissue binding. Second, when sections thicker than 200 µm were

tested, fine details such as fibres in the deepest parts of the tissue were not resolvable using multi-photon microscopy. Therefore other techniques would be required to completely capture the length of SERT+ fibres (one possible approach is discussed later. See section 5.4)

NET+ fibre analysis:

In 200 µm coronal sections (n=5. Fig. 3.8A) the mean NET+ fibre length was higher than in 40 µm sections (n=6. Fig. 3.6 and table 3.2) and this difference is significant (Mann-Whitney test $p<0.01$). In 200 µm sections, short or punctate and radially oriented fibres were still seen so the greater mean length relates to increased numbers of longer fibres (200 µm sections: 150-300 µm: ~20%; >300 µm: ~3% in contrast to 40 µm sections: 150-300 µm: ~5%; >300 µm: <1%). However, the maximum fibre length in 200 µm coronal sections was very similar to that in 40 µm coronal sections (454.7 µm and 450.55 µm respectively, table 3.1). Though there was a higher proportion of longer fibres measured in 200 µm coronal sections, suggesting measurements from 40 µm underestimated the number of longer fibres, the mean and median fibre lengths measured in 200 µm coronal sections are still short enough to confirm that all NET+ fibres will be restricted within zones and most are likely to be restricted to innervating a single microzones (with estimated widths of ~300 µm. for review see Apps and Hawkes, 2009). Additionally, the mean length of NET+ fibres in the parasagittal plane (40 µm section thickness) was much longer than those in both the 40 µm and 200 µm coronal sections and the maximum length was much higher (723.41 µm for parasagittal in comparison to coronal: 450.55 µm (40 µm) and 454.7 µm (200 µm), table 3.2) confirming a strong anisotropy with prominent rostral-caudal trajectory for the noradrenergic afferents.

Finally, 200 µm parasagittal sections from two animals were examined with NET immunohistochemistry (Fig. 3.8B. The small sample precluded statistical analysis), qualitative examination of NET+ fibres in 200 µm parasagittal sections showed that long fibres are of a similar length to those seen in 40 µm sections. Several fibres were measured at >600 µm but none were longer than 800 µm. This indicates that measurements taken from 40 µm parasagittal sections are a good estimate of the maximum length of NET+ fibres. This indicates that individual NET+ fibres have a terminal field covering between 700-800 µm in the parasagittal plane.

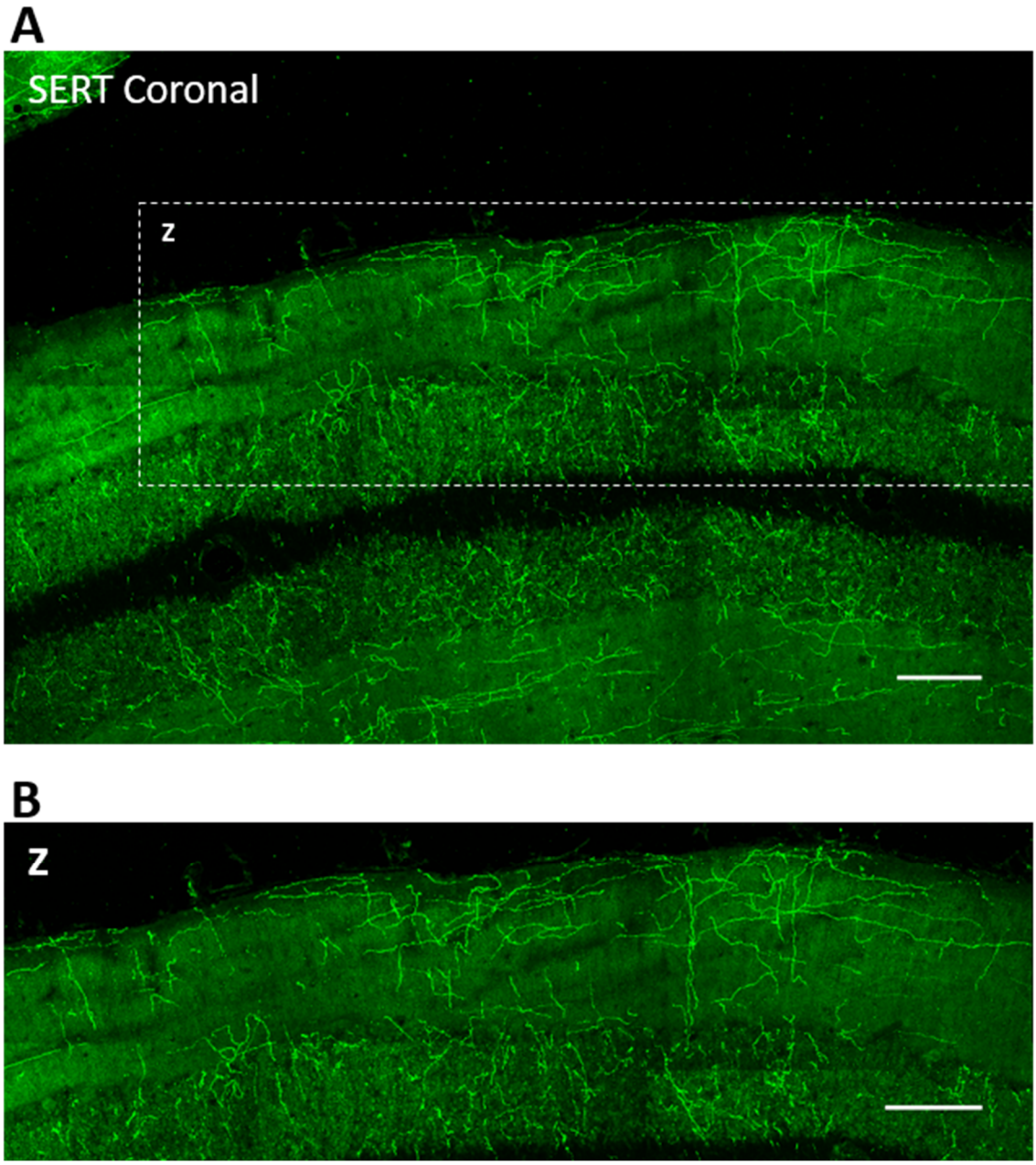


Fig. 3.7: Substantial differences in SERT+ fibre lengths are measured in 200 µm coronal sections compared to those measured in 40 µm coronal sections. A and B) In 200 µm coronal sections the mean and maximum measured fibre lengths for a SERT+ fibre was greater than those measured in 40 µm sections and the proportion of long fibres was higher. This suggests that the number of long, tangentially-oriented SERT+ fibres is underestimated in 40 µm sections. Scale bars: 150 µm.

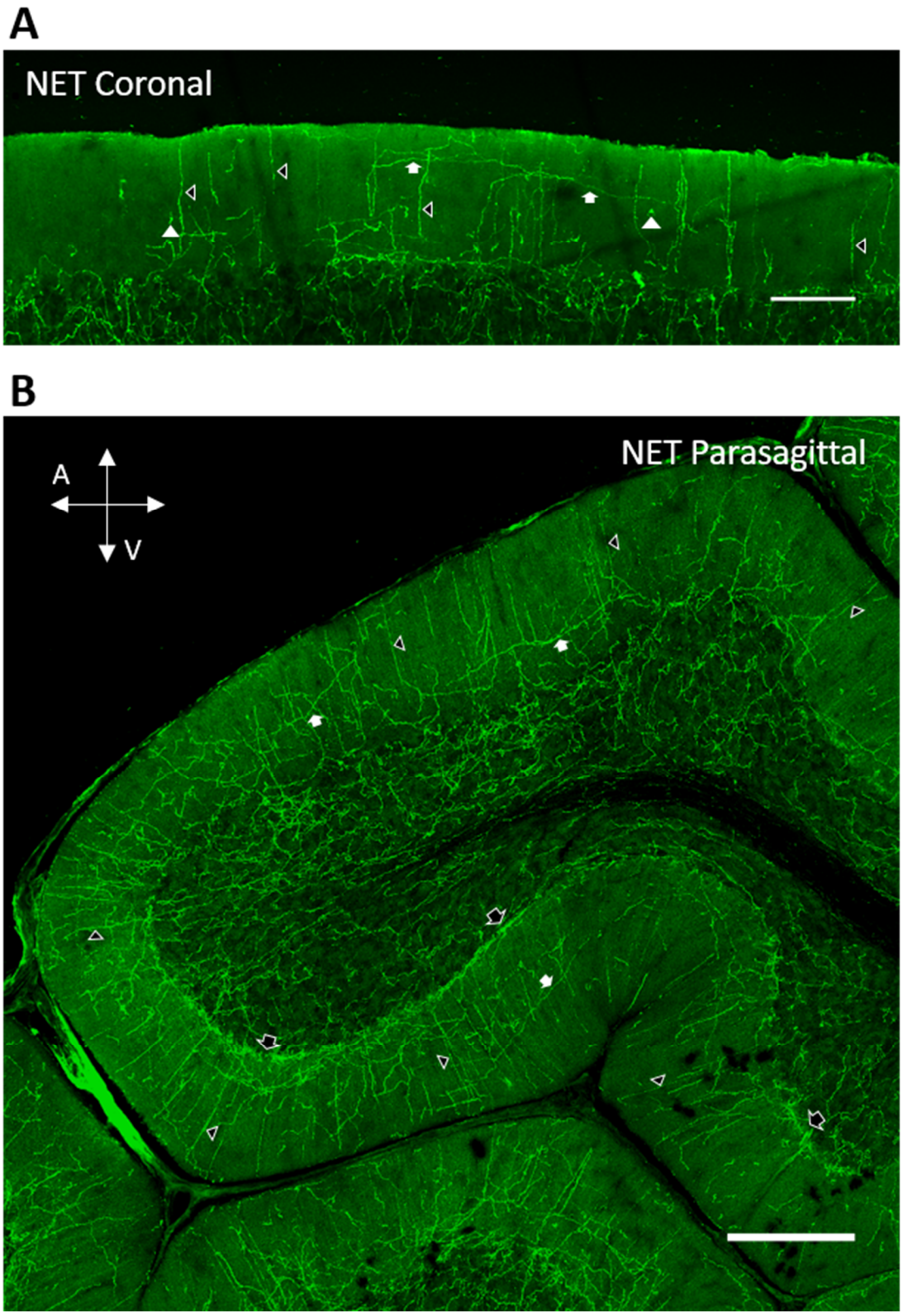


Fig. 3.8 – Legend overleaf

Fig. 3.8: NET+ fibre extents in 200 µm coronal and 200 µm parasagittal sections. **A)** In 200 µm coronal sections the maximum measured fibre length for a NET+ fibre was similar to that measured in 40 µm sections but the mean fibre length is greater and the proportion of >100 µm fibres was higher. Measurement of NET+ fibre lengths in 40 µm coronal sections leads to a small underestimate of fibre spread in the medial-lateral plane. **B)** In 200 µm parasagittal sections, the length of NET+ fibres measured was similar to the length measured in 40 µm sections so the lengths of parasagittal oriented NET+ fibres are well captured in 40 µm sections. Scale bars: A. 150 µm and B. 200 µm

3.4 Discussion

The general distribution pattern of noradrenergic and serotonergic fibres observed here is in agreement with the earlier but limited observations using a range of different methodologies (Hökfelt and Fuxe, 1969; Pickel et al, 1974; Chan-Palay, 1975; Kimoto et al, 1981; Takeuchi et al, 1982; Bishop and Ho, 1985; Felten et al, 1986). But our systematic analysis of parasagittal and coronal sections at the midline reveals, for the first time, strong anisotropies in both the noradrenergic and serotonergic afferent projections within the molecular layer. Molecular layer noradrenergic afferents have strong extensions in the rostral-caudal plane but have restricted distribution in the medial-lateral plane. In contrast, molecular layer serotonergic afferents travel long distances in the medial-lateral plane with very short extensions in the rostral-caudal plane. This strongly orthogonal anisotropy has important implications for our understanding monoaminergic influences on information processing in the cerebellar cortex.

3.4.1 SERT+ fibre distribution suggests that 5-HT acts as a diffuse signal capable of influencing multiple functional cortical microzones simultaneously

Schweighofer et al (2004) speculate that 5-HT afferents signal locally to sets of spatially separate microzones that cooperate in ongoing movement selection and learning. These afferents provide an instructive signal to coordinate the output of disparate regions and modulate shared plasticity processes in the selected microzones. On the basis of this proposal, individual 5-HT fibres might be expected to have restricted medial-lateral spread with branching to sets of functionally related microzones. However, the current findings do not support this proposal. SERT fibres extend over large distances in the medial-lateral plane. Evidence strongly suggests that serotonergic fibres release 5-HT from multiple sites along their length, so it is highly unlikely that 5-HT can select sets of microzones that collaborate to control specific movements or sets of movements. Such microzones are usually spatially separated, often on different lobules (reviewed in Apps and Garwicz, 2005). The architecture of serotonergic afferent projections in the molecular layer is consistent with the suggestion that all adjacent microzones along the medial-lateral trajectory of the fibre would be modulated. 5-HT appears to provide a global signal, modulating processing in adjacent microzones, and possibly across zonal boundaries, simultaneously.

Kerr and Bishop (1991) have previously suggested that the serotonergic projection to the cerebellar cortex is topographically organised; they observed labelling of distinct sets of serotonergic nuclei after injection of retrograde tracer in different regions of the cerebellar cortex. But each tracer infusion encompassed several lobules so the mapping was not highly detailed. Only 5-15% of neurons labelled in several source nuclei were serotonergic and these were intermingled with a mixed population of cerebellum-projecting non-serotonergic neurons. These non-serotonergic projections to the cerebellum from source nuclei that include the LRN and paramedian reticular nuclei are most likely mossy fibres, so the 5-HT signal from these sources may act in conjunction with related signals through the MF system. Schweighofer et al (2004) suggested that the 5-HT afferents from different source nuclei may act to modulate distinct sets of microzones within the cortex. However, the intermingling of 5-HT afferent source neurons with MF afferent source neurons in different brainstem nuclei is consistent with the suggestion that information through both routes might be distributed in a similar way. It is notable that the striking similarity of the parallel fibre and serotonergic afferent fibre trajectories in the molecular layer could lead to similar spatial distributions of information from the two sources across multiple microzones. The functional implications of this distribution remain to be discovered, but the recognition that the target of these inputs is a functionally heterogeneous set of Purkinje cells means that the selection and co-ordination role, as proposed by Schweighofer et al (2004), is not strongly supported.

3.4.2 NET+ fibre distribution suggests noradrenaline provide a targeted signal to the cerebellar cortex restricted to a microzone

In addition to their suggestions for 5-HT functions, Schweighofer et al (2004) also proposed that the NA signal is broadcast globally in the cerebellar cortex acting to gate the formation long-term plasticity under specific, behaviourally relevant circumstances, similar to the role proposed by Gilbert (1975). If NA were to act as a global, permissive signal we might expect to observe NA fibres that cross large regions of the cerebellar cortex, and the projection might include fibres oriented in parallel to the long axis of the folium so as to traverse the parasagittal functional compartments of the cerebellar cortex. The strong restriction of fibre trajectories in the medial-lateral plane that was observed here is entirely inconsistent with that earlier proposal and the approximation of individual, molecular layer noradrenergic afferent distributions to the area of a microzone appears more suitable for local signalling restricted to individual microzones.

3.4.3 Do individual noradrenergic afferents supply individual microzones in the cerebellar cortex?

The distribution of NET+ fibres in the ML and probably GCL is restricted in the medial-lateral plane. The dimensions of their distribution match microzone dimensions so they are capable of signalling to a single microzone. The average medial-lateral extent of individual NET+ fibres are similar in size to the medial-lateral spread of individual CFs measured by Sugihara et al (2001) in the rat, supporting the indication that NET+ fibres are restricted to individual microzones. This restricted distribution is in contradiction to the traditional view that the noradrenaline modulates all target regions simultaneously. This view was developed on the basis of some studies that found neurons in the LC with projections to more than one brain structure, for example two studies where large paired injections of tracer were made in the cerebellum and forebrain observed a proportion of labelled cells in the LC that were double labelled: ~50% (Steindler, 1981) and ~15% (Nagai et al, 1981). However, no studies have examined the organisation of noradrenergic afferents *within* the cerebellum. In two recent studies, using more restricted tracer injections in functionally distinct regions of the frontal cortex: the orbitofrontal, medial prefrontal, anterior cingulate cortex (Chandler et al, 2013) or these three regions and the primary motor cortex (Chandler et al, 2014) only between 1-5% of labelled neurons in the LC were double labelled. This suggests that within terminal structures the noradrenergic system may also be capable of providing restricted modulation to functionally distinct compartments, this may extend to individual microzones or functionally associated microzones.in the cerebellum

Additionally, the LC neurons that project to different target regions would need to be independently activated so that they could provide distinct modulation to restricted microzones in the cerebellum. No study has examined the activity of cerebellar projecting LC neurons, but Chandler et al (2014) showed the expression of proteins related to synaptic excitability and synaptic transmission, the spontaneous firing frequencies and responsiveness to glutamate were different for medial prefrontal, orbitofrontal cortical and primary motor cortex projecting neurons. The authors suggested these differences would allow neurons that projected to the different regions of the frontal cortex to be independently recruited and this independently modulate their target regions. The authors focused their examination on differences in glutamatergic signalling mechanisms. However, other studies have demonstrated intrinsic organisation of the LC that supports the view that noradrenergic afferents could differentially modulate distinct functional targets; Corteen et al (2011) showed that

distinct cell-types in different LC compartments have different patterns of $GABA_A$ subunits, indicating that subpopulations of LC neurons can be differently modulated by GABAergic as well as glutamatergic signalling. Additionally, Loughlin et al (1986a, b) showed that neurons in the LC that modulate different regions are localised to different compartments of the LC and that they are morphologically distinct, suggesting distinct functional properties.

3.4.4 Conclusion

The implications of the observations in this experiment are in sharp contrast to the predictions of the theory of Schweighofer et al (2004) regarding noradrenergic and serotonergic afferent distribution in the cerebellar cortex. Specifically the observation that 5-HT afferents project across large regions of the cortex in the medial-lateral plane indicates 5-HT signalling influences multiple microzones simultaneously and could potentially bridge boundaries between zones. Additionally, even the 200 µm sections we used here would not capture the full extent of the curvature of the folium, consequently it is likely that the medial-lateral extent of serotonergic fibres is much longer then measured here and serotonergic fibres may signal across multiple zones. The observation that NA afferents are restricted in their medial-lateral extent but cover a much larger area in the rostral-caudal plane indicates that NA signalling may influence specific microzones. Recent evidence supports the view that LC neurons can independently modulate different functional regions.

The quantification of fibre lengths was restricted to the molecular layer in the present study. For noradrenergic afferents a similar fibre pattern was also observed in the GCL as the ML, indicating the rostral-caudal restriction seen in the ML could be an organizational principle of the noradrenergic projection in the cerebellar cortex. However, the high density of noradrenergic fibres in the GCL in parasagittal sections precluded accurate distinction of fibres for measurement. In contrast, serotonergic fibres in the GCL and PCL were of a similar orientation whether they were viewed in parasagittal or coronal sections, indicating they have no specific orientation in these layers. This observation is consistent with the proposal by Takeuchi et al (1982) that the serotonergic fibres project radially through the GCL and PCL to reach their terminal fields that are restricted to the ML.

Chapter 4: β_1-adrenoceptor mediated consolidation mechanisms in the cerebellar cortex

4.1 Introduction

4.1.1 The cerebellar cortex is critical for the consolidation of nictitating membrane response conditioning

Classical conditioning of the nictitating membrane response (NMR) in rabbits and the closely linked eye-lid blink response (EBR) are critically dependent on the function of the cerebellum (McCormick and Thompson, 1984; Yeo et al, 1985a, b; Aksenov et al, 2004; 2005; Bracha, 2004; Garcia and Mauk, 1998; Garcia et al, 1999; Gruart et al, 1997; Hesslow et al, 1994a; Hesslow et al, 1999; reviewed in Longley and Yeo, 2014).

Many models of cerebellar-learning propose that the critical site of memory storage is the cerebellar cortex and that the essential mechanism is a change in the response of PCs to particular patterns of MF input (Marr, 1969; Albus, 1971; Ito, 1972; Gilbert, 1974; Hesslow and Yeo, 2002). By restricting pharmacological interventions to the post-training period, it has been revealed that the cortex, but not nuclei, is the site of a critical, time-dependent consolidation process in NMR conditioning, confirming the proposal that a significant component of cerebellar learning is stored in the cerebellar cortex (Attwell et al, 2002b; Cooke et al, 2004; Kellett et al, 2010). These earlier studies disrupted consolidation of NMR conditioning using the $GABA_A$ agonist muscimol (Attwell et al, 2002b; Cooke et al, 2004; Kellett et al, 2010) indicating that the consolidation process is sensitive to levels of $GABA_A$ activation. Various forms of cerebellar cortical plasticity identified *in vitro* could be candidate mechanisms for the storage of this learning (Hansel et al, 2001; Boyden et al, 2004; De Zeeuw and Yeo, 2005) but the mechanism *in vivo* is yet to be identified (but see section 4.1.3 below). It is possible that the cortical consolidation signal in NMR conditioning directly influences the $GABA_A$ receptor but muscimol has previously been shown to disrupt acquisition and performance of NMR conditioning (Hardiman et al, 1996; Attwell et al, 2002b; Cooke et al, 2004; Kellett et al, 2010) so a second, more likely possibility is that muscimol infusions disrupt general cortical excitability or signalling mechanisms. Thus,

there may be a cortical state in which the consolidation signal cannot execute its normal function.

4.1.2 Noradrenaline provides a specific consolidation signal in the cerebellar cortex

Gilbert (1975) proposed a role for the noradrenergic input to the cerebellar cortex as a third signal in cerebellar learning, together with CF and MF signals as proposed in the Marr-Albus models (Marr, 1969; Albus, 1971). Gilbert (1975) proposed that NA acts as a consolidation signal in cerebellar learning. Studies that have made pre-training infusions of β-adrenoceptor antagonists support the importance of noradrenergic activation of β-adrenoceptor in cerebellum-dependent learning tasks, including NMR/EBR conditioning and VOR adaptation (Pompeiano et al, 1991; Bickford, 1993; 1995; Heron et al 1996; Gould, 1998; Cartford et al, 2002; and see Cartford et al, 2004b for a review). Pre-training pharmacological interventions cannot differentiate between effects on memory acquisition processes and consolidation but post-training interventions can. Paredes et al (2009) blocked EBR conditioning with post-training infusions of the β-adrenoceptor antagonist propanolol, consistent with a role for NA in consolidation, although some of the effects may have resulted from non-specific effects of propanol and some from spread of the antagonist to the nuclei. Kellett and Yeo (2007) disrupted the consolidation of NMR conditioning with post-training cortical infusions of the β-adrenoceptor antagonist atenolol. The consolidation impairments were seen only when the infusions were in lobule HVI and if atenolol was infused within the 2-hour consolidation time window (Cooke et al, 2004). The better confinement of atenolol to the cerebellar cortex and its lack of non-specific effects confirmed a specific role for noradrenaline as a cortical consolidation signal.

Propanolol, as used by Paredes et al (2009) is relatively non-selective for β_1- and β_2-adrenoceptors. Atenolol, as used by Kellett and Yeo (2007) is usually considered as moderately selective for β_1-adrenoceptors but, when used as local infusions *in vivo* effective concentrations are difficult to define, so an action on β_2-adrenoceptors cannot be ruled out. As has been described in Chapter 2, there are distinct differences in the cellular expression of β_1- and β_2-adrenoceptors in the cerebellar cortex, so determining the relative contribution of each β-adrenoceptor type to consolidation of NMR conditioning will have important implications for our understanding of the consolidation signal and its mechanisms. If the consolidation is exclusively mediated by β_1-

adrenoceptors then the NA-mediated effects are localised to the Purkinje cell. A β_2-adrenoceptor specific activation would indicate a novel glial mechanism for memory consolidation.

We have started the investigation of the NA signalling mechanisms by examining the effect of post-training cortical infusions of the highly-selective β_1-adrenoceptor antagonist Betaxolol on consolidation of NMR conditioning.

4.1.3 Is performance of the conditioned response controlled by $mGlu_7$-mediated inhibition the Purkinje Cell?

In addition to understanding the mechanisms that control acquisition and consolidation, there are remaining questions about the control of the learned response expression. These two aspects of cerebellar learning will probably be linked, as consolidation and expression mechanisms must co-operate during the extended training periods. The conditioned suppression of the PC has been shown by Hesslow et al (1999) and Jirenhed et al (2007) to be related to performance of the conditioned EBR. Two recent studies (Johansson et al, 2014; 2015) have revealed that this conditioning-like suppression of PC firing is not controlled by glutamatergic or GABAergic activation of ionotropic receptors on PCs but by activation of the metabotropic $mGlu_7$ receptor. Because the relationship between conditioned PC suppression and the conditioned EBR is highly likely to be causal, this finding suggests that *in vivo* activation of the $mGlu_7$ receptor controls performance of the CR. If this is the case, then the β-adrenoceptor controlled consolidation process may act on the expression, or functional activation, of $mGlu_7$ receptors in PCs or modulate intracellular targets of the $mGlu_7$ signalling pathway.

We tested whether $mGlu_7$ activation is important for performance of the behavioural, conditioned NMR, as it is for conditioned PC suppression. We made cortical infusions of the selective $mGlu_7$ antagonist MMPIP after conditioning had been established to test its effect on the expression of CRs.

4.1.4 NMR conditioning is critically dependent upon a specific region of Lobule HVI

As discussed in Chapter 1 (section 1.2.7) there is evidence for an eye-blink control region at the base of lobule HVI that is critical for the acquisition, consolidation and performance of NMR conditioning (Yeo et al, 1985b; Hardiman and Yeo, 1992; Yeo and Hardiman, 1992; Hardiman et al, 1996; Attwell et al, 1999, 2001, 2002a, b; Cooke et al, 2004; Kellett et al, 2010; Mostofi et al, 2010) and EBR conditioning (Hesslow et al, 1994a; Hesslow et al, 1999; Jirenhed et al, 2007; Halverson et al, 2015; Ohmae and Medina, 2015; ten Brinke et al, 2015). Mostofi et al (2010) used field potential recordings to localise a D0 eyeblink microzone, with a likely accompanying C3 microzone, in the rabbit using criteria defined by Hesslow (1994a, b) in the original studies. The region was localised to ventromedial lobule HVI, adjacent to lobule V but separated from it by the primary fissure. In some instances, the eyeblink microzone was seen to reach the depths of the primary fissure. Whether the microzone extended for a short distance along the lateral wall of lobule V was difficult to assess because of its proximity to the HVI component on the other wall of the primary fissure. The field potentials were weaker in lobule V, indicating that their source may have been in lobule HVI. The conclusion was that the majority of the eyeblink microzone territories are in lobule HVI in the rabbit.

However, some studies in rabbits have claimed that an intact lobule HVI is not necessary for conditioned NMR performance (Perret et al, 1993) or EBR conditioning and extinction (Perret and Mauk, 1995; Garcia et al, 1999) but instead lobules IV/V of anterior lobe are critical (Garcia et al, 1999 and Green and Steinmetz, 2005). The smallest effective anterior lobe lesion reported in Garcia et al (1999) included regions of lobule V immediately adjacent to HVI.

In the present study, the aim was to test the effects upon consolidation of a selective β_1-adrenoceptor antagonist and of a selective $mGlu_7$ antagonist upon expression of conditioned responses. This was to be achieved by infusion cannula placements adjacent to the critical eyeblink control region in lobule HVI, as in previous studies (e.g. Attwell et al 2002b; Kellett et al 2010). Here, changes of availability required that a new strain of rabbits was used. This new strain had minor morphological differences in the anatomy of these regions of the cerebellum, so several of the cannula placements

were within lobule V. Thus, some analysis of the contribution of lobule V and lobule HVI to the learning was possible.

4.1.5 Experimental summary

Post-training intracortical infusions of the specific betaxolol were used to test the role of β_1-adrenoceptor activation in consolidation of classical conditioning of the NMR. Pre-training intracortical infusions of the specific $mGlu_7$ antagonist MMPIP were used in trained subjects to test the role of $mGlu_7$ activation in the performance of the conditioned NMR.

4.2 Methods

4.2.1 Animals

Adult male New Zealand white rabbits (approximately 2.5-3kg) were used. All procedures were approved by the local ethical review panel of the University of Cambridge and were in accordance with the UK Home Office Animals (Scientific Procedures) Act. All subjects were housed individually, allowed food and water ad libitum, and maintained on a 12 hr light/dark cycle for at least 2 weeks before surgery.

4.2.2 Surgery

Pre-operative treatments:

Subjects were anaesthetised with fentanyl/ fluanisone (0.315/10 mg ml; Hypnorm 0.4 ml kg i.m.) supplemented with benzodiazepam (0.2 – 0.4 mg kg i.v.) and intubated with a lubricated endotracheal tube and given an intravenous infusion of mannitol (20% w/v, 30 ml over 20 min) before surgery.

Surgery:

Rabbits were placed in a stereotaxic instrument, with bregma set at 4.2 mm below lambda. Anaesthesia was maintained with isoflurane (in 33% N_20, 66% O_2. 3-5% for induction, 1.5-2.5% for maintenance). Depth of anesthesia was monitored by the stability of respiratory rate and the absence of withdrawal reflexes. Under sterile conditions, the scalp was reflected laterally and a small craniotomy (~1.5 cm in diameter) was made with a dental drill to expose part of the right cerebellar cortex. The dura was cut and reflected, and cerebellar lobule HVI identified by visual inspection. A 26G stainless steel cannula (Plastics One, Roanoke, VA; length 15 mm below pedestal) was inserted 4-5 mm below the surface of the medial lobule of HVI and fixed to the skull using cyanoacrylate and dental acrylic. The scalp was sutured around the implant, and a 33G dummy cannula was used to seal the guide cannula. Postoperatively, subjects received saline (0.9 %; 20 ml kg s.c.), an antibiotic (enrofloxacin; 5 mg kg s.c.) and analgesic (buprenorphine; 0.05 mg kg i.m.). Gas anaesthesia was withdrawn, the scalp incision region was cleaned and the subjects

were allowed to recover in a quiet environment before being returned to the home cage.

Post-operative treatments:

Subjects were maintained on 3 days of antibiotic (enrofloxacin, 5 mg kg s.c.) and analgesics (buprenorphine, 0.05 mg kg i.m). In their home cages, all subjects were housed individually, allowed food and water ad libitum, and maintained on a 12 hr light/dark cycle. Behavioural testing began after a minimum period of one week after surgery.

4.2.3 Conditioning protocols

Subjects were placed in a custom-made Perspex restraining stock and a monofilament loop was sutured in the right nictitating membrane under local anesthesia (proxymetacaine hydrochloride, 0.5% w/v). An isotonic potentiometer was secured to the head at the ears and muzzle and secured to the NM by a hook through the monofilament loop to record the NM movement. Two stainless steel wound clips were attached to the skin, one immediately behind the temporal canthus of the eye, the other immediately below the centre of the lower eyelid for delivery of the unconditional stimuli (US).

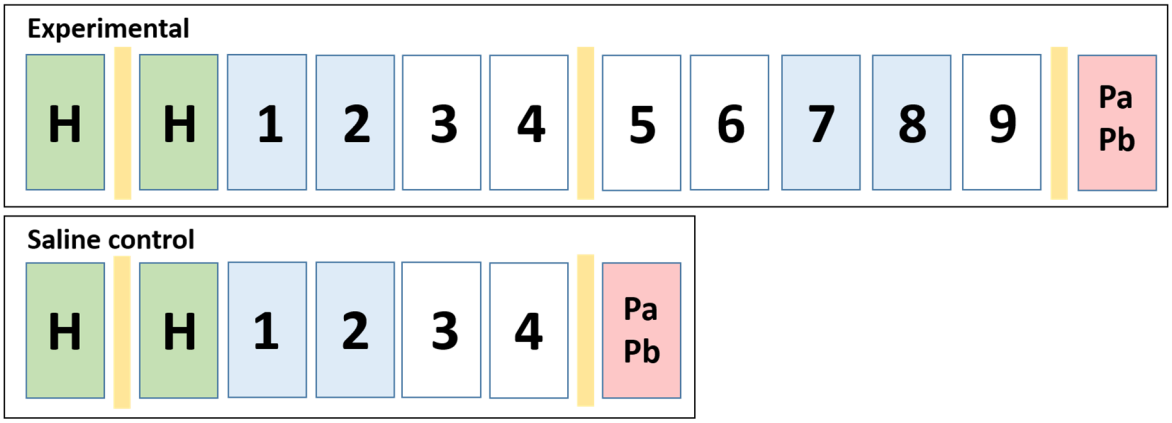


Fig. 4.1: Order of sessions for experimental and saline control subjects. Habituation sessions - green; acquisition sessions with post-training infusions - blue, without infusion – white; Performance testing sessions - Pink. 72 hour no-training period - yellow bar. Abbreviations: H – Habituation, Pa – Performance testing, session a; Pb – performance testing, session b.

Habituation session:

For habituation sessions, subjects were placed in the restraining stocks, the transducer and US delivery clips were attached and subjects were placed in ventilated, sound-attenuating (conditioning) chambers facing a centrally mounted loudspeaker. Background noise produced by ventilation fans was 58 dB. No conditional stimulus (CS) or US was delivered during habituation sessions. Subjects were given two habituation sessions of 25 minutes each to adapt to the novel environment, habituation sessions were given on two separate days separated by 72 hours.

Conditioning session:

Each conditioning session consisted of 50 trials. The (CS) was a 1 kHz sine wave tone of 410 m duration and an intensity of 81 dB (A-scale). The unconditional stimulus (US) was periorbital electrical stimulation (60 ms train of three biphasic pulses of intensity 1.0 mA) through stainless steel clips attached to previously applied wound clips. On paired trials the interstimulus interval between the CS and US onset was 350 ms. The inter-trial interval was randomly selected between 25 and 35 sec. In 45 trials the CS and US were paired, and a CS-alone trial was presented on every 10th trial. The acquisition training for experimental subjects consisted of 6 daily sessions, with a 72-hour rest between Session 4 and 5 and 4 daily sessions for infusion control subjects. Experimental subjects received a further 3 conditioning sessions to test cumulative effects of Betaxolol hydrochloride on CR performance (the order of the conditioning protocol is summarised in Fig. 4.1).

Phase 1 (Acquisition and post-training infusion. Sessions 1-2)

All subjects received two daily sessions of acquisition training. Immediately after the last trial of each session a 33 G injector was inserted in the implanted guide cannula to protrude 1.5 mm below the guide and all experimental subjects received 2 µl infusion of the highly selective β_1-adrenoceptor betaxolol hydrochloride (6 mM, n: 6) in NaCl and control subjects received 2 µl NaCl infusions (n: 5) over 2 minutes by means of a 10 µl Hamilton syringe connected to the injector with polythene tubing. The injector was left in situ for 5 min to aid drug diffusion.

Phase 2 (Acquisition/Maintenance. Sessions 3-4 control and sessions 3-6 experimental)

All subjects received two daily sessions of training with no post-training infusions. Experimental subjects received a further two daily sessions with no post-training infusions, this meant both experimental and control subjects received four daily sessions without post-training drug infusions.

Phase 3 (Maintenance)

Experimental subjects that had received betaxolol in phase 1 received two more daily sessions, each followed immediately by betaxolol infusions (as in Phase 1), followed by a final day of training with no post-training infusion. This was to assess the cumulative effects of two days of post-training administration of betaxolol on CRs.

Phase 4a (Performance Testing. CNQX)

In Phase 4, drug infusions were made pre-training to test their efficacy in disrupting expression of established CRs. These tests were used to assess whether infusions made in Phase 1 (and 3) had been in appropriate locations, and of sufficient volume to fully affect the NMR control region of HVI. All subjects received a 2 µl infusion of the non-NMDA receptor antagonist CNQX (6 mM), which has been shown to abolish CR expression when administered to the NMR control region of cortical lobule HVI, but has no effect on CRs when administered to the cerebellar nuclei. After a baseline session of 20 trials (18 paired CS–US trials and 2 unpaired CS trials) CNQX was infused immediately and a further 100 trial session was initiated (90 paired CS-US trials and 10 unpaired CS trials). The criterion to experimentally define cannula placements and infusion spread as on-target infusions was disruption of CR performance in ten consecutive trials. Subjects not meeting this criterion were considered to have received off-target infusions.

Phase 4b (Performance Testing. MMPIP)

A subset of subjects (n: 5) received a 2 µl infusion of the potent, selective $mGlu_7$ antagonist MMPIP hydrochloride (600 µM in 6% DMSO) and vehicle infusions (6% DMSO) on separate daily session in a repeated measures design, following the same performance test session structure as in phase 4a.

4.2.4 Histology

At the end of the experiment, subjects were given heparin (1000 IU i.v.) and an overdose of pentobarbitone sodium (90 mg kg i.v.), and transcardially perfused with 0.9% saline (1 litre) followed by 4% w/v paraformaldehyde (PFA) and 0.5% w/v picric acid in 0.1 M phosphate buffer. The brain was removed, post-fixed and cryoprotected in 20% sucrose/ 4% PFA solution for two days before embedding in gelatin. For embedding, the sucrose was washed from the brain by soaking under running water overnight and the overlying arachnoid matter was removed. Brains were blocked with a coronal cut just rostral to the superior colliculi and the cerebellar block was submerged in 5% gelatin for 2 hours at 50°C under 600 mbar pressure followed by submersion in 10% gelatin for 1 hour at 50°C under 600 mbar pressure. The gelatine block was allowed to set overnight and fixed in 20% sucrose/ 4% PFA solution for at least three days. Gelatin-embedded cerebellar blocks were freeze-mounted onto the stage of a sledge microtome (Leitz 1400; Leica, Germany) and cut in 50 µm serial coronal sections. Every fourth section was mounted on gelatin-subbed glass slides, air-dried on to the slides overnight then stained with cresyl violet and mounted under a glass coverslip under a non-aqueous permanent mountant (DPX. VWR, Leicestershire, U.K.). Sections were examined under a light microscope for evidence of cannula damage, gliosis, Purkinje and granule cell loss. Infusion sites were identified and reconstructed on standard transverse cerebellar diagrams.

4.2.4 Data analysis

Movement of the NM was measured by an isotonic transducer and the signal was fed into an analogue to digital converter (Micro 1401. CED LTD, Cambridge, U.K.). Baseline and within trial position of the NM was acquired using custom software (Blink, Peter Trigg, UCL). A CR was defined as an NMR within the CS–US interval with

amplitude ≥ 0.5 mm and with onset latency > 35 ms from CS onset. CR frequency (% CRs) was calculated for all trials throughout the conditioning sessions. Descriptive statistics for each subject and group averages for each session (and each 10 trial block) were automatically calculated by custom software (Bookmaker, Peter Trigg, UCL). Data analysis was performed using SPSS 19. Results of Kolmogorov-Smirnov Tests and the Shapiro-Wilk Tests revealed data was not normally distributed so for all group comparisons non-parametric tests were used.

4.2.5 Drugs & Solutions

All drugs were obtained from Tocris Bioscience (Bristol, U.K.): betaxolol hydrochloride (catalogue number: 0906), CNQX disodium salt (catalogue number: 1045), and MMPIP hydrochloride (catalogue number: 2963).

4.3 Results

4.3.1 CNQX performance test

After all subjects had received at least four daily sessions of acquisition training without drug infusion they received a 20 trial conditioning session to establish a baseline CR rate and then given an infusion of CNQX, followed immediately by a 100 trial session to test the effects on CR performance. It has previously been shown that infusions of CNQX into lobule HVI leads to a fast acting and profound disruption of CR performance and a criterion of 10 consecutive missed CRs trials has been previously used to define an on-target cannula placement (Kellett et al, 2010; Kellett and Yeo, 2007). No subject fully reached this criterion and thus no subject could be experimentally defined as having received a fully on-target infusion.

4.3.2 Post-training cerebellar cortical Betaxolol infusions had no effect on consolidation

After two habituation sessions, subjects received two conditioning sessions with post-training cortical infusions: the experimental group received betaxolol hydrochloride, a highly selective β_1-adrenoceptor and a control group received matched volumes of NaCl. The learning curves of both groups over the first four sessions were closely matched (Fig. 4.1), though there is a small trend towards a slower rate of learning in the experimental group. Using percentage of CRs (% CRs) performed in each session as a measure of acquisition there was no significant difference in % CRs in session 3 (Mann-Whitney U test, $U = 14$, df = 9, $P > 0.05$) or session 4 (Mann-Whitney U test, $U = 14$, df = 9, $P > 0.05$) between the betaxolol and control group. No firm conclusions can be drawn at this stage about the role of β_1-adrenoceptor activation in consolidation as CNQX infusion performance tests indicated no cannula placements were sufficiently close to the critical compartment in Lobule HVI.

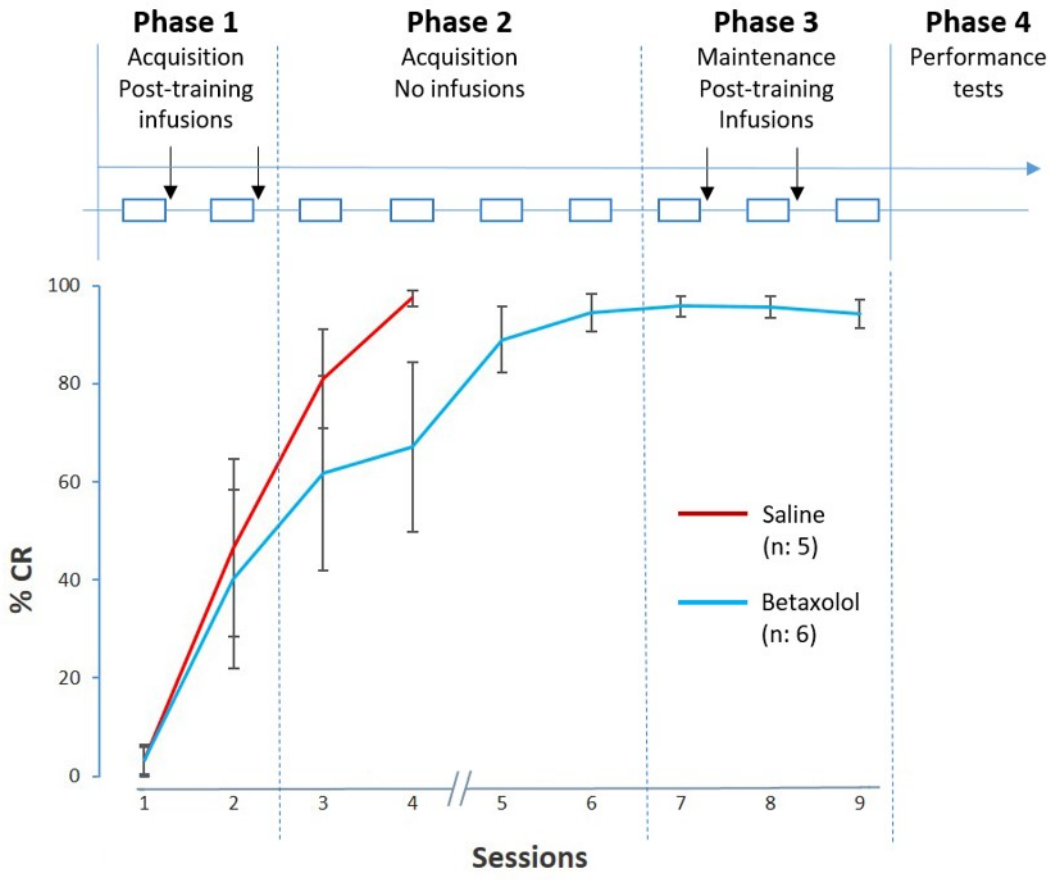


Fig. 4.2: Acquisition of CRs following two daily post-training infusions of betaxolol or saline. The frequency of conditioned responses is expressed as a % of trials per session showing a CR. Session CR frequencies are averaged across subjects in each group. The first two sessions were sessions with infusions (indicated with arrows), as were sessions seven and eight for the betaxolol group. Sessions four and five were separated by 72 hours as indicated by a gap in the x-axis. Error bars: S.E.M.

4.3.4 Examination of cannula placements reveals that regions within lobule IV/V are not essential for the expression of NMR conditioning

Upon completion of acquisition and performance experiments all subjects were perfused and their brains were removed, the cerebellum was sectioned and stained to aid histological examination. The categorisation of most subjects as off-target based on CNQX performance test was confirmed with histological examination revealing cannula placements outside of lobule HVI in all but one subject (A0107 from the betaxolol group. See Fig. 4.2, Fig. 4.4b, c, d).

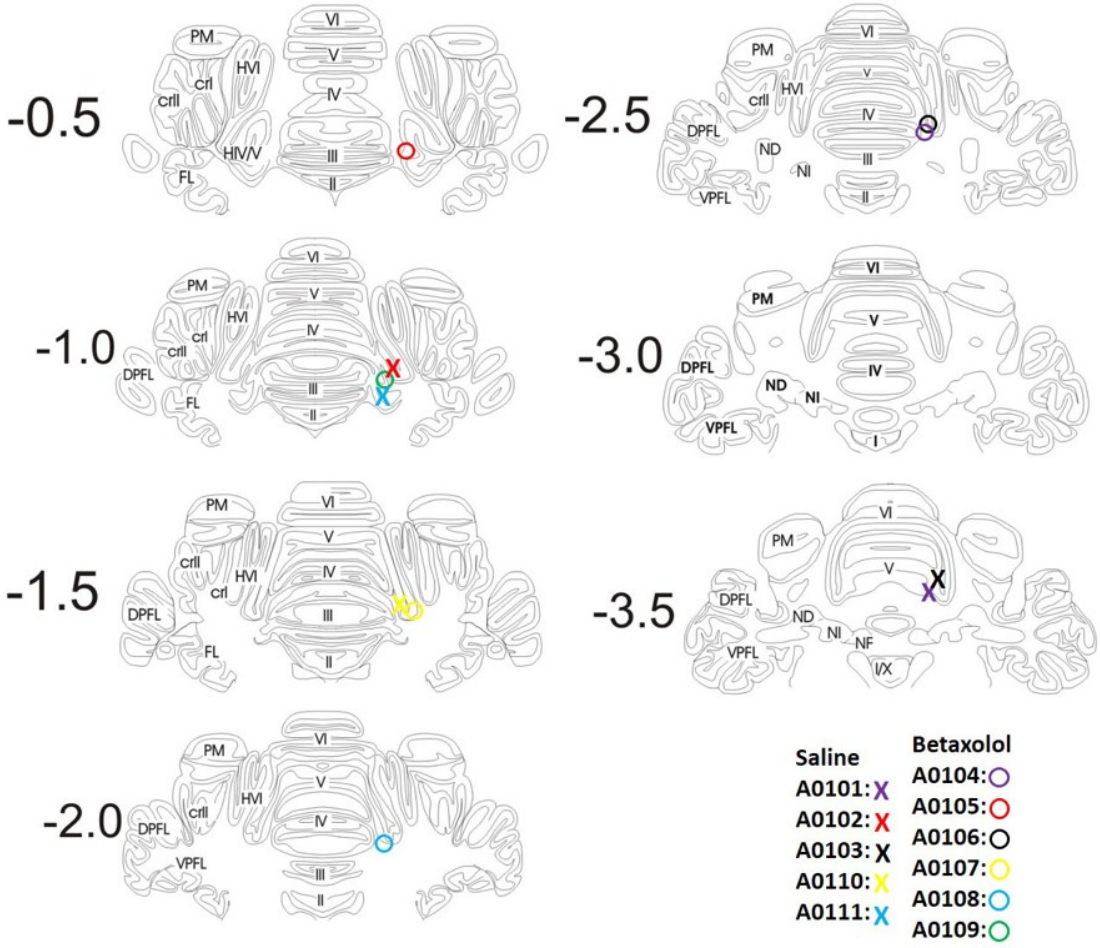


Fig. 4.3: Summary of cannula placements. Reconstruction of cannula tip position for all betaxolol (ring) and control (cross) animals. Roman numerals identify lobules by the scheme of Larsell (see Chapter 1), PM: paramedian lobule, CrI and CrII: crus 1 and 2, DPFL/ VPFL: dorsal and ventral paraflocculus, FL: flocculus, ND/NI/NF: dentate, interpositus and fastigial nucleus. Numbers refer to distance (mm) of section rostral to lamda.

Eight of eleven subjects had cannula placements in lobule IV/V: subjects A0101, A0102, A0103, A0110 from the saline group and A0104, A0106, A0108 and A0109 from the betaxolol group. (See Fig. 4.2, Fig. 4.3, Fig. 4.4). The cannula tip of one subject was localised to lobule HIV/V (A0105 from the betaxolol group). None showed significant impairment in CR performance following CNQX infusion as had been seen following CNQX infusions into lobule HVI (Attwell et al, 1999; 2001; 2002b; Cooke et al, 2004; Kellett et al, 2010 and Kellett and Yeo, 2007). This is in sharp contrast to observations by Perret et al (1993) and Garcia et al (1999) that lobules IV/V are

critically involved in NMR/EBR conditioning, including performance of conditioned NMR (Perret et al, 1993). Even CNQX infusions through cannulae very close to the critical regions of lobule IV/V identified by Garcia et al (1999, e.g. subject A0110, Fig. 4.4e, f) appeared to be no more effective at disrupting CR performance than those in lobule IV/V but far from the critical region (e.g. subject A0106, Fig. 4.4b, c). See Fig. 4.4a and 4.4d for comparison of % CR performance after CNQX infusion for subjects A0106 and A0110.

One subject (A0107) was judged to have cannula tip placement in lobule HVI. Subject A0107 was part of the betaxolol group and when the acquisition curve for A0107 is compared to the rest of the group it suggests that betaxolol infusion into the base of lobule HVI leads to disruption of conditioning (Fig. 4.5a). However, A0107 showed no performance deficit after CNQX infusion (Fig. 4.5b). Close examination of the cannula track trajectory and cannula tip location shows that the guide cannula transects most of the white matter within lobule HVI and the tip of the guide cannula has caused significant damage to the cortical layer at the medial base of lobule, where the critical NMR control regions have been localised (Mostofi et al, 2010) whilst the injector tip appears to have reached well into the underlying white matter, as indicated by gliosis (box, Fig 4.5c). Thus, a potential explanation for these contrasting effects could be that the damage to the NMR regions of lobule HVI caused by the guide were not complete but were severe enough to delay learning. Furthermore, the cannula tip was in the underlying white matter and immediately adjacent to the fourth ventricle so the CNQX infusion did not cover Lobule HVI but instead reached the underlying white matter or was drained to the fourth ventricle.

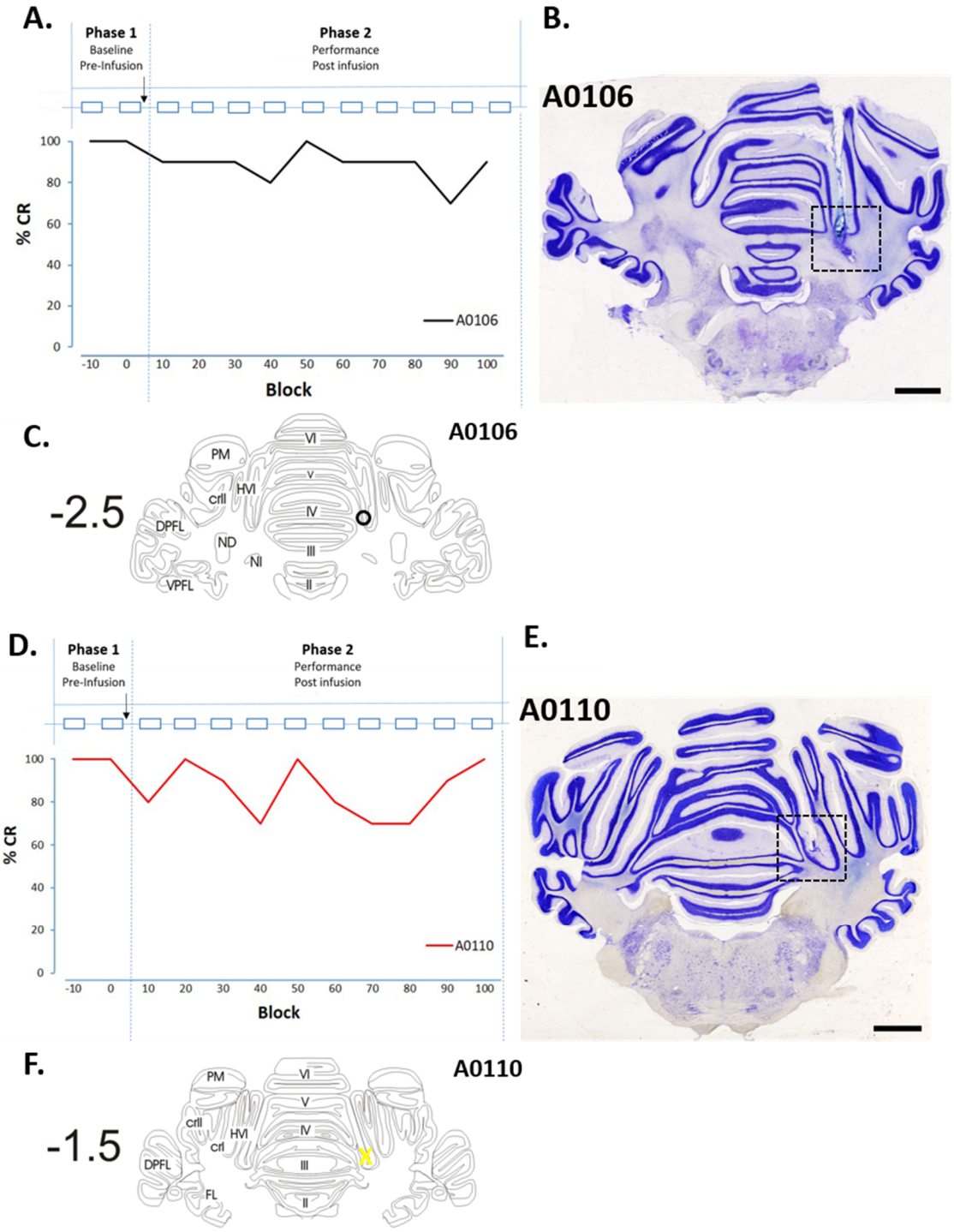
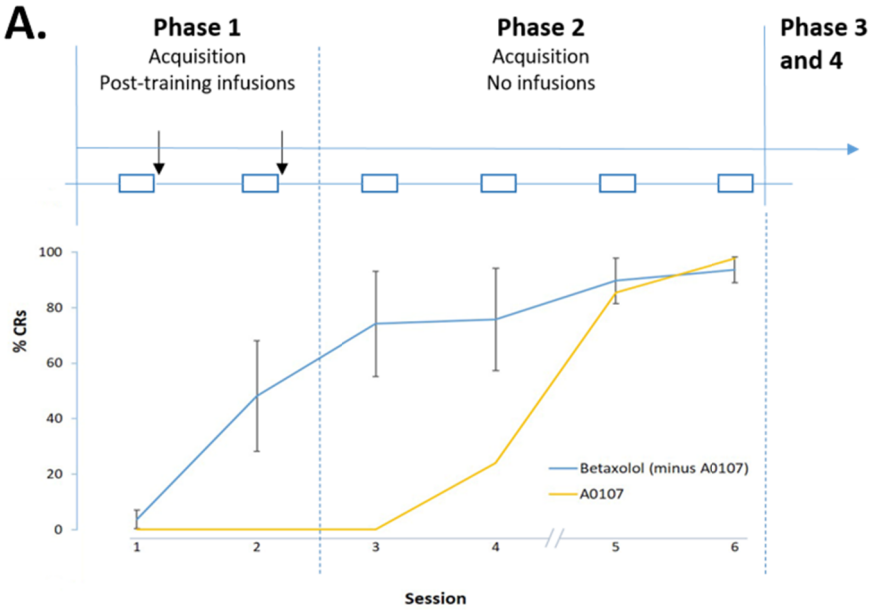
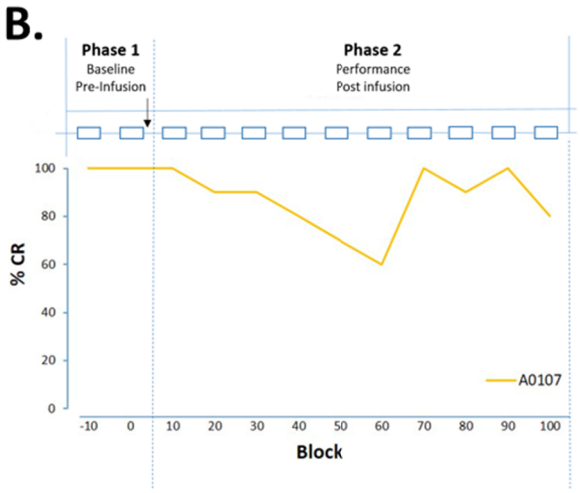
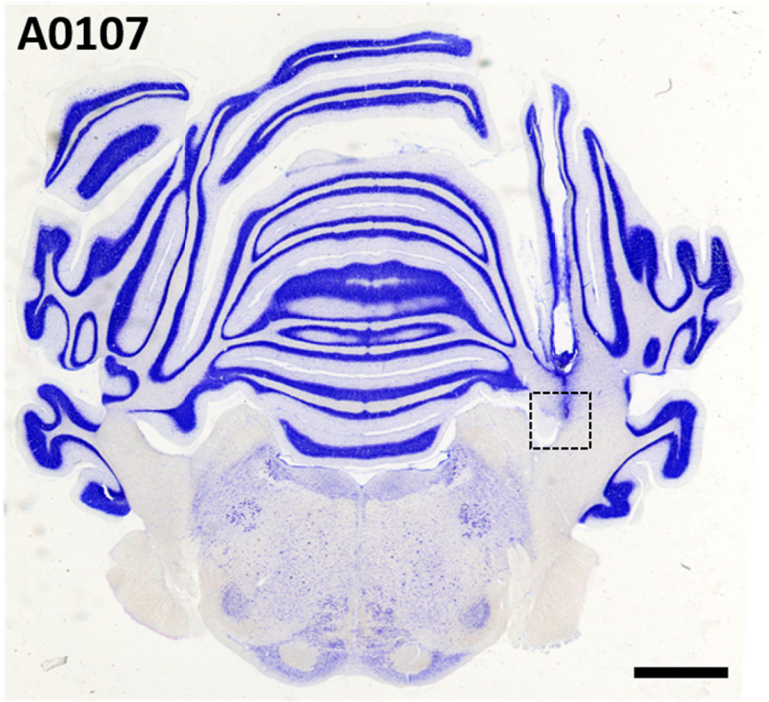


Fig. 4.4: Reconstruction of cannula placements and relationship to CNQX performance test. A and D) Percentage CRs per ten trial block for two subjects, A0106 (betaxolol) and A0110 (control) following CNQX infusion. **B and E)** Nissl stained sections, in B the cannula track can be observed dorsal to the cannula tip (outlined by box) and gliosis of underlying white matter is visible. In E the cannula track is not visible because of the plane of section but cannula tip (outlined by box) can be seen just lateral of the primary fissure. **F and E)** Reconstruction of cannula tip for each subject based on examination of sections. Scale bars: 2 mm.


A.
Phase 1
Acquisition
Post-training infusions
Phase 2
Acquisition
No infusions
Phase 3
and 4
% CRs
Session
Betaxolol (minus A0107)
A0107


B.
Phase 1
Baseline
Pre-Infusion
Phase 2
Performance
Post infusion
% CR
Block
A0107


C.
A0107

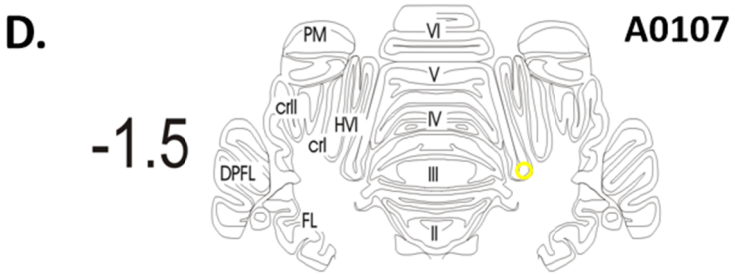

D.
A0107
-1.5
PM
VI
V
crII
HVI
IV
crI
DPFL
III
FL
II

Fig. 4.5: Reconstruction of cannula placement, acquisition curve and performance on CNQX performance test for subject A0107. A) The frequency of conditioned response session by session during acquisition training for A0107 presented with the average for the remaining Betaxolol subjects (error bars: S.E.M.) **B)** Percentage CRs per ten trial block by A0107 CNQX infusion. **C)** Nissl stained section, the cannula track can be clearly observed transecting the medial and lateral leaflet of lobule HVI and passing through the white matter, cannula tip (outlined by box) is clear and gliosis of underlying white matter caused by injector track is visible. **D)** Reconstruction of cannula tip based on examination of sections. Scale Bar: 2 mm.

4.3.3 Performance of established conditioned responses were spared by MMPIP infusion

A subgroup of five subjects (A0107-11) were used to examine the effects of MMPIP hydrochloride (600 μM) on performance of pre-established CRs. A repeated measures design was used with each subject receiving a performance test after MMPIP infusion and after infusion of vehicle (6% DMSO) on two separate days. Visual inspection of CR % for each group in the performance test shows very little deficit in performance caused by MMPIP infusion and very little difference between the two conditions (Fig. 4.6). Based on the criteria for designating a performance deficit developed for lobule HVI CNQX infusions, MMPIP infusion did not lead to a performance deficit, with no subject failing to perform CRs on ten consecutive trials. There was also no significant difference in % CRs over the course of the performance test between MMPIP and vehicle infusions (Wilcoxon Signed Ranks, $Z = 1.461$, $df = 5$, $P = 0.144$). No strong conclusion can be drawn from this result as to the involvement of $mGlu_7$ activation in the performance of the conditioned NMR as performance testing with CNQX and histological analysis revealed no subject had an on-target cannula placement. When successful HVI placements are tested, these results will, however, be useful in pointing to any possible specificity in localisation of the $mGlu_7$-mediated effects.

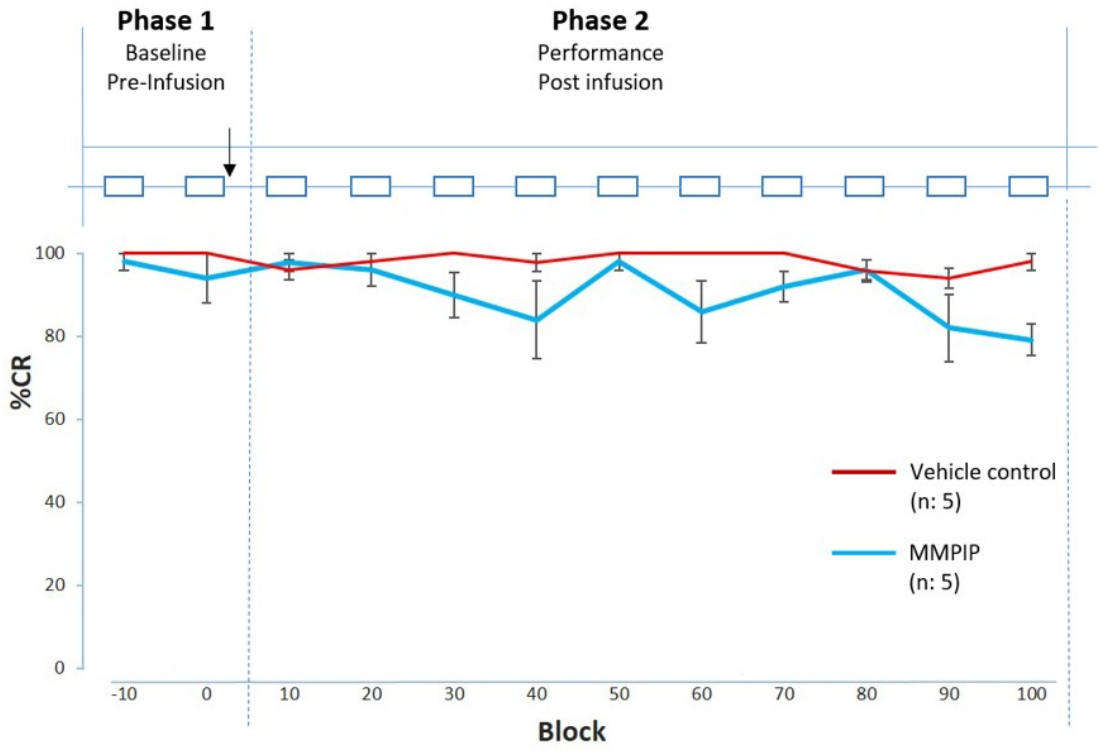


Fig. 4.6: Effect of MMPIP on CR performance. Percentage CRs per ten trial block for subjects following infusions of MMPIP or vehicle. A repeated measures design was used (N: 5) with each subject receiving MMPIP and vehicle on two separate daily sessions. (error bars: S.E.M.)

4.4 Discussion

4.4.1 The requirement for β-adrenoceptor and mGlu$_7$ activation in consolidation and performance of NMR conditioning

Post-training infusions of the selective β_1-adrenoceptor did not produce impairments in the consolidation of NMR conditioning in this study and pre-session infusions of the selective mGlu$_7$ antagonist MMPIP did not disrupt execution of established CRs. However, based on the effects of CNQX on CR performance and post-mortem histological localisation of injector tips in all subjects it was concluded that infusion sites were not placed sufficiently close to the critical NMR region in ventromedial lobule HVI for the infused drugs to modulate essential activity. As a result, no firm conclusions can be drawn as to the involvement of β_1-adrenoceptor or mGlu$_7$ activation in consolidation and performance of NMR conditioning, respectively.

Further experimentation to define the role of β_1- and β_2-adrenoceptor activation in consolidation and the mGlu$_7$ in expression of NMR conditioning *in vivo* should be a priority for future research. Since its discovery by Masao Ito and colleagues, PF-PC LTD has been the main candidate cellular mechanism for cerebellar learning (Ito et al, 1982; Ito and Kano, 1982). Despite the identification of a number of other cerebellar plasticity mechanisms *in vitro* (Hansel et al, 2001; Boyden et al, 2004) and intensive investigation of the behavioural consequences of disrupting these different forms of plasticity (e.g. Aiba et al, 1994; Koekkoek et al, 2003; Schonewille et al, 2011), as yet there has been no consistent demonstration of the requirement of any single plasticity mechanism in cerebellum-dependent learning. If β_1-adrenoceptor or β_2-adrenoceptor and mGlu$_7$ activation are important mechanisms for consolidation and expression of learning *in vivo,* this would provide important constraints on identifying plasticity mechanisms *in vitro* as candidate mechanisms for cerebellar learning. These ideas will be discussed in further detail in Chapter 5.

4.4.2 Intact lobule IV/V signalling is not required for the performance of the classically conditioned NMR

This study used experimental animals from a source different from that used in previous studies in the host laboratory (Kellett and Yeo, 2007; Kellett et al, 2010; Mostofi et al, 2010). Due to important variations in gross anatomy of the cerebellar

cortex in the animals used here, the surface landmarks and depth measurements used in previous studies to target the infusion cannula to ventromedial HVI were not exactly matched to the subjects in this study. Consequently, the infusion cannulae tips in all but one subject (A0107) were just outside lobule HVI and the cannula of subject A0107 was deep so that the injector tip cleared the base of HVI. More accurate stereotaxic coordinates for this new strain of rabbits have now been developed, however they could not be implemented in an experimental group in time for inclusion in this study.

The majority of cannula tip placements in this study were localised to lobule IV/V (9/11 subjects), therefore the lack of disruption of CR performance observed following CNQX infusion suggests that lobule IV/V is not critical for the performance of conditioned NMRs. Mostofi et al (2010) confirmed that the critical control region for the NMR and conditioned NMR responding is located in the ventromedial lobule HVI with some part of the critical region extended around the base of the primary fissure and encroaching on lobule V, as a result of between-subject anatomical variation. The findings of Mostofi et al (2010) and others (Yeo et al, 1985a, b, c; Hardiman and Yeo, 1992; Attwell et al, 1999; Kellett et al, 2010) confirm the anatomical and functional similarities between the NMR control region and the eye-blink control region identified by Hesslow and colleagues (Hesslow, 1994a, b; Hesslow et al, 1999; Jirenhed et al, 2007) and underline the restricted nature of the critical region in relation to the entire cortical territory of lobule HVI. These results confirm the lack of involvement of signalling in lobule IV/V to NMR conditioned responding even in regions of lobule V in close anatomical proximity to ventromedial lobule HVI, confirming the importance of functional identification of eye-blink or NMR control regions in electrophysiological studies examining conditioning related changes in cerebellar activity (Longley and Yeo, 2014). Recent studies that have not used the criteria defined by Hesslow (1994a, b) to confirm the eye-blink controlling nature of the recorded PCs have made inconsistent observations as to the nature of the PC change in conditioning (Halverson et al, 2015; Ohmae and Medina, 2015; ten Brinke et al, 2015). For example, Green and Steinmetz (2005) observed heterogeneous changes in PC firing following EBR conditioning when recording from lobule IV/V. These findings stand in contrast to the consistent observation of suppression of PC activity during conditioned responding where the recorded PCs are in the C3 eyeblink microzone (Jirenhed et al, 2007)

4.4.3 Conclusion

This study was unable to assess the involvement of the β_1-adrenoceptor or $mGlu_7$ receptor in lobule HVI in cerebellar learning processes. Future studies using the newly derived stereotaxic coordinates obtained here will need to be completed to test these hypotheses. However the subjects from the current study will be important in establishing localisation effects within the full study. The finding that none of the subjects who had cannula placements located in lobule IV/V (9/11) showed performance deficits after CNQX infusion confirms that intact signalling in this region is not required for conditioned NM responding.

Chapter 5: General Discussion

Noradrenaline and serotonin are the neuromodulators in two prominent afferent systems within the cerebellum. Noradrenaline signalling via activation of β-adrenoceptors is critical for consolidation of cerebellum-dependent, classical conditioning of the NMR/EBR (Kellett and Yeo, 2007; Paredes et al, 2009). 5-HT fibres comprise the third largest afferent system in the cerebellum (Kerr and Bishop, 1991) and there is evidence that systemic infusions of 5-HT_{2A} antagonists disrupt acquisition of EBR conditioning (reviewed in Harvey, 2003). Despite evidence for the importance of both systems in cerebellar function, examination of their roles within the cerebellar circuit has been neglected. Here I will summarise the key findings of the thesis (section 5.1) and discuss the implications for cerebellar learning and future empirical work (section 5.2, 5.3, 5.4)

5.1 Summary of results

Chapter 2:

Chapter 2 presents the first detailed examination of β_1-, β_2- and α_1-adrenoceptor expression in the cerebellar cortex. The regional distribution and cell specific localisation of each receptor type was mapped. Expression of all three adrenoceptor subtypes was present across all regions of the cerebellar cortex, suggesting a consistent role for each in cerebellar processing. The cell-specific expression was different for each subtype. β_1- and β_2-adrenoceptor expression was highly restricted and distinctive in PCs and BGCs respectively. α_1-adrenoceptor expression was more heterogenous, with expression in PCs, GoCs and MLIs. The implications of these distinct distributions are discussed below (section 5.2).

Prompted by the observation in earlier studies that infusions of a β-adrenoceptor antagonist, in sufficient volumes to modulate cerebellar cortical and nuclear processing, disrupt acquisition and consolidation of cerebellum-dependent learning (Cartford et al, 2002; 2004a; Paredes et al, 2009) the distribution of β_1- and β_2-adrenoceptor expression in the cerebellar nuclei was also examined. Both β-adrenoceptors were expressed in each of the individual cerebellar nuclei. The lack of cell-specific immunohistochemical markers for cerebellar nuclei neurons precluded a fully detailed, cell specific analysis of expression. But the distribution of β-adrenoceptor

immunoreactivity by somata size revealed β-adrenoceptors expression in cells of all size ranges. However, β_1-adrenoceptors are present only in approximately 40% of small neurones, a percentage much smaller than for β_2-adrenoceptor expression. These observations suggest that β_1-adrenoceptors expression may be limited in the small nuclear interneuron sub-class.

The widespread cerebellar nuclei expression of β-adrenoceptors complicates the interpretation of previous studies (e.g. Cartford et al, 2002; 2004a and Paredes et al, 2009) because the disruption to cerebellum-dependent learning may, in part, have resulted from spread of propranolol to the cerebellar nuclei. Kellett and Yeo (2007) used techniques that limited drug effects upon the cerebellar nuclei, but such effects cannot be completely excluded. Future studies may need to examine the effects of local infusions of β-adrenoceptor antagonists into the cerebellar nuclei on NMR conditioning acquisition and consolidation.

Chapter 3:

The distributions of MF and CF afferents within the cerebellar cortex have directly inspired the leading theories and models of cerebellar function. The 5-HT and NA afferent systems are claimed to be two of the largest neuromodulatory systems in the cerebellum so their absence in many theoretical treatments suggests that a detailed account of their distribution within the cortical circuitry, for implementation in more detailed models, is long overdue. In Chapter 3, the distribution of the serotonergic and noradrenergic afferents was examined. Both afferent types were present across the entire cerebellar cortex. When the distribution of both afferents was examined in relation to the organisation of microzones and zones, contrasting anisotropies were observed. Serotonergic afferents covered extensive areas in the medial-lateral plane but were relatively restricted in the rostral-caudal plane, noradrenergic afferents ran orthogonal to the serotonergic afferent distribution (Fig. 5.1B). The functional implications of these distributions is discussed below (see section 5.4).

Chapter 4:

Previous studies have shown that local infusions into lobule HVI of the β-adrenoceptor antagonist atenolol prevent consolidation of NMR conditioning (Kellett and Yeo, 2007). Prompted by the observation here that β_1- and β_2-adrenoceptors are expressed by different cell types in the cerebellar cortex, and that β_1-adrenoceptor expression is unique to PCs, Betaxolol, a β_1-adrenoceptor specific antagonist, was used to examine the role of β_1-adrenoceptor activation on consolidation. Additionally, following the observation that application of MMPIP, a specific $mGlu_7$ antagonist, blocks expression of conditioned pauses in PC firing rate in a reduced analogue of NMR/EBR conditioning, the effects of intracortical MMPIP infusions on behavioural CR expression were examined.

Due to technical limitations no infusions were effectively targeted to lobule HVI, thus the possible involvement of β_1-adrenoceptor and $mGlu_7$ activation in NMR conditioning was not fully tested. However, the majority of subjects received infusions of CNQX targeted to lobule IV/V, a region that has previously been implicated in NMR/EBR conditioning (Perret et al, 1993; Perret and Mauk, 1995; Garcia et al, 1999), contrary to these studies no involvement of lobule IV/V in the expression of CRs was seen.

5.2 Functionally important distinctions in the expression of adrenoceptors in the cerebellar cortex

The sharp distinction in expression of each the adrenoceptors examined here has implications for the potential mechanisms of a noradrenergic consolidation signal.

β-adrenoceptors:

Activations of β_1- and β_2-adrenoceptors have acute effects on cell signalling (reviewed in Salgado et al, 2016 and Marzo et al, 2009), including in the cerebellar cortex where they have been shown *in vitro* to potentiate the response of PCs to GABAergic signalling or to potentiate GABAergic signalling onto PCs (Yeh and Woodward, 1983; Cheun and Yeh, 1992; Mitoma and Konishi, 1999; Saitow et al, 2000a, b; Saitow et al,

2005). It has been shown that suppression of PC activity is related to CR performance in EBR conditioning (Jirenhed et al, 2007) suggesting the possibility that NA activation of β-adrenoceptors on PCs may be an underlying mechanism in CR expression. However, the results of present study looked specifically at the effect of disrupting β-adrenoceptor activation on consolidation, with the results indicating that β-adrenoceptor activation is at least partly involved in controlling long-term changes to the cortical circuitry that underpin cerebellar learning.

Both β-adrenoceptor types are Gs-coupled metabotropic receptors that initiate production of adenylate cyclase, which in turn controls the activation of the cAMP-PKA pathway that can mediate the activation of CREB. CREB is a transcription factor and its activation leads to changes in protein synthesis, which are suggested to underpin memory consolidation (see Kandel et al, 2014 for review). The cAMP-PKA-CREB pathway has been shown to be important for induction of LTP in the hippocampus (Frey et al, 1993; Huang and Kandel, 1996; Walling and Harley, 2004) and activation of this pathway, including by β-adrenoceptors, is critical for learning *in vivo* (Vianna et al, 2001; Havekes et al, 2012; Zhou et al, 2013). In analysis of cerebellar learning, Cartford et al (2004a) made pre-training infusions of Rp-cAMPS, an inhibitor of cAMP activation of PKA, into the lobule HVI of rats (though the volume of infusion was designed to also reach the underlying interpositus nucleus) and EBR conditioning was impaired. Presumably, the effect on PKA activation of the drug infused pre-training would have overlapped with the consolidation period identified by Cooke et al (2004). This study identifies β-adrenoceptor activation of PKA signalling pathways as a potential candidate to mediate acquisition and/or consolidation processes.

If β_1-adrenoceptor activation mediates the consolidation signal this would be consistent with the proposal by Gilbert (1975) that noradrenergic signalling on to the Purkinje cell controls consolidation of cerebellar learning. Additionally, it would provide a possible link between NA as a consolidation signal and a PC-specific $mGlu_7$ mechanism for expression of cerebellar learning (Johansson et al, 2015) if the $mGlu_7$ mechanism is confirmed as important to behavioural expression of CRs (discussed in section 5.3)

If β_2-adrenoceptor activation mediates the noradrenergic consolidation signal, then this would implicate BGCs as the mediator of consolidation in cerebellum-dependant learning. As reviewed in Chapter 2 (section 2.4.2) BGCs express a range of

neurotransmitter receptors and are activated by a range of neurotransmitters and motor behaviour including, locomotion-induced modulation of BGCs that is partly adrenoceptor-mediated (Nimmerjahn et al, 2009; Paukert et al, 2014). If the β_2-adrenoceptor were to mediate a specific consolidation signal, this indicates it might initiate a long-lasting change in BGCs. AMPAR receptor expression in BGCs is enriched in the lateral appendages that ensheathe PF, CF and MLI synaptic contacts on PCs (Castejón et al, 2002). When Saab et al (2012) made selective, inducible knock-outs of BGC AMPA receptor expression in juvenile and adult mice, they observed retraction of the lateral appendages. In adult, but not juvenile mice the loss of lateral appendages correlated with disruption of the acquisition and performance of EBR conditioning. Thus, structural plasticity of the lateral appendages and consequent modulation of synaptic signalling on to PCs represents a mechanism through which β_2-adrenoceptor activation on BGCs might underpin learning.

Structural plasticity of astroglial ensheathments of synapses (including lateral appendages of BGC) as a means of long-term modulation of neuron-neuron signalling is a growing area of interest (Bernardinelli et al, 2014). Structural plasticity of ensheathments can modulate neural signalling in several ways; acting as a physical barrier it can alter the surface area of pre- and post-synaptic regions available for signalling and alter the rate of diffusion of neurotransmitter from the synapse, thus altering the magnitude and dynamics of synaptic and extra-synaptic signalling (Theodosis and Poulain, 1993; Piet et al, 2004). Glial ensheathments can also actively modulate timing and magnitude of synaptic signalling by expression of reuptake-transporters and neurotransmitter recycling enzymes (Derouiche and Frotscher, 1991; Oliet et al, 2001; Piet et al, 2004) and they may exert some control over the maintenance of dendritic spines (Nishida and Okabe, 2007). These changes in synaptic signalling may also bias the type and magnitude of plasticity that occurs at neuron-neuron synapses (Panatier et al, 2006. all mechanisms reviewed in Bernardinelli et al, 2014).

α_1-adrenoceptor:

The observation that the α_1-adrenoceptor is expressed on many cerebellar cortical cells, including PCs suggests that it may also play a critical role in cerebellar processing, which may include cerebellar learning mechanisms. One of the

downstream intracellular mediators of α_1-adrenoceptor activation is PKC (Protein Kinase C. Marzo et al, 2009), activation of this kinase is a critical element in PF-PC LTD induced *in vitro,* the most prominent candidate mechanism for cerebellum-dependent learning *in vivo* (intracellular signalling mechanisms reviewed by Ito, 2001; 2002). However, an early study found no effect of systemic α_1-adrenoceptor blockade on rotarod running, a putative cerebellum-dependent learning task (Watson and McElligot, 1984; Heron et al, 1996), but the special reliance of this task on the cerebellum has not been established using the same controlled experimentation as for classical conditioning of NMR and EBR and VOR adaptation. It will be important to examine the role of α_1-adrenoceptors on cerebellar learning using tasks with confirmed cerebellar dependence.

5.3 The expression of β-adrenoceptors in the cerebellar cortex and their activation in consolidation: Implications for plasticity and memory storage mechanisms

The Marr (1969) and Albus (1971) theories propose that cerebellar learning is controlled by coincident MF (via PFs) and CF input to the PC. The main candidate cellular mechanism for cerebellar learning has been PF-PC LTD which occurs following coincident stimulation of PF and CF input to the PC *in vitro* and *in vivo* (Ito et al, 1982; Ito and Kano, 1982; Ekerot and Kano, 1985; Sakurai, 1987; Karachot et al, 1994, see Ito, 2002 for review). There has been mixed success in confirming the reliance of behaviour on this cellular mechanism. Some empirical support for PF-PC LTD as the learning mechanism comes from studies that disrupted cerebellum-dependent learning with pharmacological or transgenic manipulations of molecular mechanisms that mediate PF-PC LTD (Aiba et al, 1994; Boyden et al, 2006; De Zeeuw et al, 1998; Feil et al, 2003; Hansel et al, 2006; Koekkoek et al, 2003). However a more recent study using molecular techniques for targeted disruption of upstream signalling in the LTD cascade (Schonewille et al, 2011) revealed that behavioural learning survives disruption of LTD using these techniques.

Recent observations suggest the vast majority of PF-PC synapses may be ‘silent’ under naive conditions (Isope and Barbour, 2001; Ekerot and Jorntell, 2001; Jorntell and Ekerot, 2002) and that baseline PC activity is generated intrinsically (Hausser and Clarke, 1997). Where cerebellar learning is expressed by a suppression of PC activity, PF-PC LTD alone might not underlie cerebellar learning because the further

depression of an already silent population may not provide the necessary range and pattern recognition ability thought to be necessary. Furthermore, it is not clear how depression of a relatively small number of PF-PC synapses would lead to the strong PC firing rate suppressions seen in recent studies. Finally, a range of different forms of plasticity have been identified *in vitro,* and some *in vivo*, throughout the cerebellum (for example Sakurai, 1987; Salin et al, 1996; D'Angelo et al, 1999; Jorntell and Ekerot, 2003; Belmeguenai and Hansel, 2005; Jorntell et al, 2010). It has been suggested that any of these other plasticities may contribute to cerebellar learning (Hansel et al, 2001; Boyden et al, 2004 and Schonewille et al, 2010). For example Dean et al (2010; Dean and Porrill, 2014) have proposed a dual pathway account of cerebellar learning. In addition to plasticity of the direct excitatory PF-PC synapse, plasticity at the indirect inhibitory PF-MLI synapse is critical to cerebellar learning in their adaptive filter model of cerebellar function.

Recent work has suggested a solution to questions about learned PC firing rate suppressions. A novel role has been identified for activation of PC $mGlu_7$ receptors in controlling conditioned suppression of PC activity, in a reduced *in vivo* analogue of EBR conditioning (Johansson et al, 2015). Johansson et al (2016) propose a specific theory for how $mGlu_7$ activation controls the conditioned suppression. They have previously shown that a well-timed suppression can be initiated by a time invariant PF signal (Johansson et al, 2014), on this basis they suggest that activation of $mGlu_7$ receptors expressed on a CS-onset specific pattern of synapses initiates a signalling cascade that controls a change in PC excitability at a particular latency. This latency is determined by the presence of a particular array of intracellular biochemical components and membrane-bound ion channels that are downstream targets of $mGlu_7$ activation, all of which can be regulated by learning.

Pharmacological manipulation of cerebellar cortical functions in NMR conditioning have revealed a time-limited consolidation process that is critically dependent on noradrenaline (Cooke et al, 2004; Kellett and Yeo, 2007). Few *in vitro* studies of cerebellar plasticity have considered noradrenergic modulation and none over the behaviourally relevant time window. This may explain why studies that have made targeted disruptions of plasticity mechanisms identified *in vitro* have failed to prevent learning *in vivo* (Schonewille et al, 2011). The preparation of cerebellar slices for *in vitro* study may compromise monoamine afferents and leave them non-functional so that *in vitro* preparations lack normal monoaminergic input, including the critical

noradrenaline signal. The failure to compensate for a loss of monoaminergic signalling in the slice may lead to an incomplete representation of the plasticity mechanisms present *in vivo.* A second possibility is that monoaminergic afferents remain fully functional *in vitro* but the stimulation protocols used to study plasticity trigger abnormal monoamine release.

For in vitro analyses of cerebellar learning, the glutamatergic signalling of parallel fibres and climbing fibres has traditionally been considered to mediate plasticity mechanisms. The failure to recognize the status of monoaminergic signalling *in vitro* could lead to an incomplete representation of cerebellar plasticity mechanisms. Our behavioural experiments suggest that future *in vitro* work will need to consider the state of monoaminergic signalling in the slice and interpret the data accordingly.

Behavioural studies of memory often examine learning processes over the course of hours, e.g. Cooke et al (2004) showed that muscimol sensitive consolidation processes occur for up to two hours post-training. However most *in vitro* studies examine plasticity over the course of seconds to minutes. By confining examination of the molecular mechanisms of plasticity to relatively short time windows *in vitro* studies may overlook critical mechanisms. Based on the observations presented here and in Kellett and Yeo (2007) the molecular mechanisms of cerebellar learning are likely to develop over the course of two hours, to be localised to the Purkinje cell or Bergmann glia cell and to be regulated by noradrenaline activation of β_1- or β_2-adrenoceptors (or both). Future *in vitro* studies will need to consider both the time frame over which molecular mechanisms of plasticity are examined and the condition of monoaminergic signalling in the slice when interpreting the relevance of the results to *in vivo* learning processes.

The recent findings by Johansson et al (2015) that have identified a role for $mGlu_7$ receptors in controlling the conditioned suppression of PC activity presents a highly likely possibility for an interaction between the consolidation of cerebellar memories and their expression. The specifics of this theory are still to be empirically tested but the finding here that PCs express β_1-adrenoceptors, suggests that if β_1-adrenoceptor activation mediates consolidation, the downstream signalling cascades initiated by β_1-adrencoeptor activation could act on $mGlu_7$ receptors directly or on targets of $mGlu_7$ activated signalling. Therefore, examination of the specific involvement of the β_1-adrenoceptor and $mGlu_7$ activation in NMR conditioning is a critical avenue of research

to understand possible cellular mechanisms of cerebellar learning. Unfortunately, we were unable to complete this analysis in the work presented in Chapter 4 but it will be the subject of future research.

5.4 The distribution of NA and 5-HT afferents: clues to function

The observations reported in Chapter 3, that the distribution of individual noradrenergic afferent fibres approximates the area of an individual microzone suggests that the noradrenergic projection to the cerebellum could independently modulate distinct functional regions of the cerebellar cortex. This is in contrast to theories of noradrenergic signalling that suggest the LC broadcasts a uniform signal throughout the brain, that modulates levels of behavioural arousal and controls orientation of attention (Berridge and Waterhouse, 2003; Sara and Bouret, 2012). However, the majority of studies on which these theories are based have examined broad noradrenergic projections to the cerebral cortex and limbic forebrain structures, especially the influence of LC signalling on the cerebral cortex and thalamic nuclei (Berridge and Waterhouse, 2003) and the prefrontal cortex (Sara and Bouret, 2012). Recent findings by Chandler et al (2013; 2014) indicate that there are groups of widely projecting neurons in the LC, but also distinct sub-sets that have very restricted terminal targets. This restricted target type of projection this could be the principle upon which cerebellar projecting noradrenergic neurons are organised.

Future studies will be needed further to elucidate functions of the noradrenergic afferent projection to the cerebellar cortex. Because of the density of NET+ fibres labelled using IHC it was not possible to follow the trajectory of a single fibre through serial sections. To trace individual, cerebellar cortical noradrenergic projections throughout their full extent, anterograde labelling of neurons in the LC and noradrenergic sub-nuclei would be a useful approach. In studies by Wu et al (1999) and Sugihara et al (2001) very small injections of biotinylated dextran neurons into the LRN and inferior olive labelled only a small sub-set of source neurons and axons, this allowed the authors to trace the entire cerebellar trajectory of a single MF or CF, respectively. This approach, if possible would provide fuller insights into the distribution of noradrenergic afferents across the cerebellar cortex and would allow the distribution of a single noradrenergic afferent in relation to anatomical markers of function, such as Zebrin II compartments, to be defined.

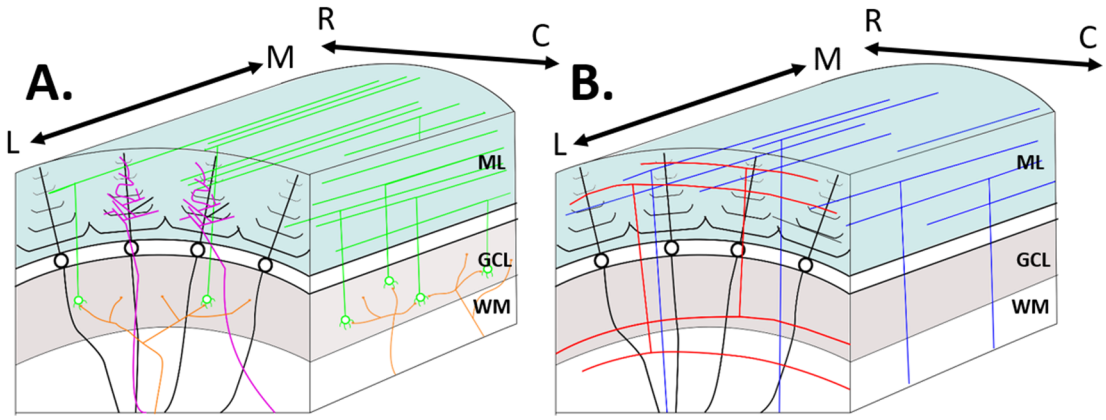


Fig 5.1: Illustration of three-dimensional arrangement of noradrenergic and serotonergic afferent fibres in the molecular layer (B) with arrangement of climbing fibres and parallel fibres for comparison (A). A) Diagram from midline vermis, so medial-lateral is parallel to the long axis of the folia and rostral-caudal is perpendicular (orientations denoted by arrows). Climbing fibres (purple) and the Purkinje cell dendritic arbors (black in ML) lie perpendicular to the folia whilst parallel fibres (green) run along the folia. Also shown, the mossy fibre afferents (orange) contacting the granule cells that give rise to the parallel fibres. **B)** Noradrenergic afferents (red) run perpendicular to the long axis of the folia whilst serotonergic afferents (blue) run along the folia. ML: molecular layer, GCL: granule cell layer, WM: white matter, M: medial, L: lateral, R: rostral and C: caudal.

The orientation of the serotonergic afferents was shown to be similar to that of parallel fibres and in complete contrast to that of the noradrenergic afferents, suggesting different functional capacities. The observation that serotonergic fibres extend up to 900 µm in the medial-lateral plane is likely to be an underestimate of the true extension of these fibres. It was very common that the start and finish of a fibre measurement was determined not by the ends of the fibres but instead by the upper and lower surface limits of the section. This is to be expected if the serotonergic fibres travel the long-axis of the folium in a manner similar to PFs. The curve of the long axis of the folium is likely to exit the plane of section at short distances from the midline, even in 200 µm sections. As suggested above for noradrenergic fibres, anterograde labelling of serotonergic afferents from their source nuclei using small infusions of tracer may be a useful method for examining the full extent of serotonergic afferents, as the fibres could be traced more easily through serial sections.

The widespread nature of the 5-HT fibre distribution suggests that 5-HT signals a global modulatory signal. On the basis of the effects of systemic 5-HT_{1A} antagonist and agonist application on hippocampal-dependent learning it has been suggested that serotonergic signalling in the hippocampus provides a negative gating signal that holds off learning processes (reviewed in Ogren et al, 2008 and Meneses and Lily-Salmeron, 2012). This may be a possibility for serotonergic signalling in the cerebellum, however, the observations from Harvey and colleagues (reviewed in Harvey, 2003) that 5-HT_{2A} activation enhances acquisition of EBR conditioning suggests that in the cerebellum serotonergic 5-HT signalling may positively modulate learning. It may be that 5-HT has a similar role to the one suggested for NA by Schweighofer et al (2004), providing a global signal that gates plasticity so that it only occurs under selected behavioural circumstances. This suggestion is supported by the observation that activity of the serotonergic source nuclei and 5-HT levels in the cerebellum correlate with behavioural state (Veasey et al, 1995; Mendlin et al, 1996). The experiments by Harvey and colleagues (Harvey, 2003) used systemic infusions of 5-HT_{2A} ligand to implicate 5-HT signalling in conditioning. Future studies could make pre-training localised infusions to lobule HVI during acquisition or performance of NMR conditioning to test specifically for a role of cerebellar cortical 5-HT_{2A} activation in cerebellum-dependent learning.

5.5 Summary

The cell specific distribution of β_1-, β_2- and α_1-adrenoceptors in the cerebellar cortex has been shown. The distinct distribution of the β_1- and β_2-adrenoceptor has important implications for cellular mechanisms of cerebellar learning, the contribution of each to consolidation of NMR conditioning and their possible interaction with $mGlu_7$ mediated mechanisms of CR expression are a critical area of future study. The widespread expression of α_1-adrenoceptors in the cerebellar cortex warrants further exploration with behavioural techniques. The widespread expression of β-adrenoceptors in the cerebellar nuclei indicates a potential separate contribution of β-adrenoceptor function in cerebellum-dependent behaviour, which could be explored with localised infusions. Finally, the observation of distinct patterns of noradrenergic and serotonergic afferent distribution in the cerebellar cortex indicates each make different contributions to cerebellar cortical processing. Anterograde tracing of the afferent fibres may be a fruitful approach to defining the exact nature of their functions in relation to known organisational principles of the cerebellum.

Bibilography

ACOSTA-MARTINEZ, M., FIBER, J. M., BROWN, R. D. & ETGEN, A. M. 1999. Localization of alpha1B-adrenergic receptor in female rat brain regions involved in stress and neuroendocrine function. *Neurochem Int,* 35**,** 383-91.

AGNATI, L. F., ZOLI, M., STRÖMBERG, I. & FUXE, K. 1995. Intercellular communication in the brain: Wiring versus volume transmission. *Neuroscience,* 69**,** 711-726.

AIBA, A., KANO, M., CHEN, C., STANTON, M. E., FOX, G. D., HERRUP, K., ZWINGMAN, T. A. & TONEGAWA, S. 1994. Deficient cerebellar long-term depression and impaired motor learning in $mGluR_1$ mutant mice. *Cell,* 79**,** 377-388.

AKSENOV, D., SERDYUKOVA, N., IRWIN, K. & BRACHA, V. 2004. GABA neurotransmission in the cerebellar interposed nuclei: involvement in classically conditioned eyeblinks and neuronal activity. *J Neurophysiol,* 91**,** 719-27.

AKSENOV, D. P., SERDYUKOVA, N. A., BLOEDEL, J. R. & BRACHA, V. 2005. Glutamate neurotransmission in the cerebellar interposed nuclei: involvement in classically conditioned eyeblinks and neuronal activity. *J Neurophysiol,* 93**,** 44-52.

ALBUS, J. S. 1971. A theory of cerebellar function. *Mathematical Biosciences,* 10**,** 37.

ALTMAN, H. J., NORDY, D. A. & OGREN, S. O. 1984. Role of serotonin in memory: facilitation by alaproclate and zimeldine. *Psychopharmacology (Berl),* 84**,** 496-502.

ANDERSSON, G., GARWICZ, M. & HESSLOW, G. 1988. Evidence for a GABA-mediated cerebellar inhibition of the inferior olive in the cat. *Exp Brain Res,* 72**,** 450-6.

ANDERSSON, G. & HESSLOW, G. 1987a. Activity of Purkinje cells and interpositus neurones during and after periods of high frequency climbing fibre activation in the cat. *Exp Brain Res,* 67**,** 533-42.

ANDERSSON, G. & HESSLOW, G. 1987b. Inferior olive excitability after high frequency climbing fibre activation in the cat. *Exp Brain Res,* 67**,** 523-32.

ANDERSSON, G. & OSCARSSON, O. 1978. Climbing fiber microzones in cerebellar vermis and their projection to different groups of cells in the lateral vestibular nucleus. *Exp Brain Res,* 32**,** 565-79.

ANDRE, P., POMPEIANO, O. & WHITE, S. R. 1993. Activation of muscarinic receptors induces a long-lasting enhancement of purkinje cell responses to glutamate. *Brain Res,* 617**,** 28-36.

AOKI, C., GO, C. G., VENKATESAN, C. & KUROSE, H. 1994. Perikaryal and synaptic localization of alpha 2A-adrenergic receptor-like immunoreactivity. *Brain Res,* 650**,** 181-204.

APPS, R. & GARWICZ, M. 2005. Anatomical and physiological foundations of cerebellar information processing. *Nat Rev Neurosci,* 6**,** 297-311.

APPS, R. & HAWKES, R. 2009. Cerebellar cortical organization: a one-map hypothesis. *Nat Rev Neurosci,* 10**,** 670-81.

ARMSTRONG, D. L., HAY, M. & TERRIAN, D. M. 1987. Modulation of cerebellar granule cell activity by iontophoretic application of serotonergic agents. *Brain Res Bull,* 19**,** 699-704.

ARRANG, J.-M., DRUTEL, G., GARBARG, M., RUAT, M., TRAIFFORT, E. & SCHWARTZ, J.-C. 1995. Molecular and Functional Diversity of Histamine Receptor Subtypes. *Ann N Y Acad Sci,* 757**,** 314-323.

ASTON-JONES, G. & BLOOM, F. E. 1981. Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle. *J Neurosci,* 1**,** 876-86.

ASTON-JONES, G. & COHEN, J. D. 2005. AN INTEGRATIVE THEORY OF LOCUS COERULEUS-NOREPINEPHRINE FUNCTION: Adaptive Gain and Optimal Performance. *Annu Rev Neurosci,* 28**,** 403-450.

ATLAS, D., TEICHBERG, V. I. & CHANGEUX, J. P. 1977. Direct evidence for beta-adrenoreceptors on the Purkinje cells of mouse cerebellum. *Brain Res,* 128**,** 532-6.

ATTWELL, P. J., RAHMAN, S., IVARSSON, M. & YEO, C. H. 1999. Cerebellar cortical AMPA-kainate receptor blockade prevents performance of classically conditioned nictitating membrane responses. *J Neurosci,* 19**,** RC45.

ATTWELL, P. J., RAHMAN, S. & YEO, C. H. 2001. Acquisition of eyeblink conditioning is critically dependent on normal function in cerebellar cortical lobule HVI. *J Neurosci,* 21**,** 5715-22.

ATLAS, D. & MELAMED, E. 1978. Direct mapping of β-adrenergic receptors in the rat central nervous system by a novel fluorescent β-blocker. *Brain Res,* 150**,** 377-385.

ATTWELL, P. J., IVARSSON, M., MILLAR, L. & YEO, C. H. 2002a. Cerebellar mechanisms in eyeblink conditioning. *Ann N Y Acad Sci,* 978**,** 79-92.

ATTWELL, P. J., COOKE, S. F. & YEO, C. H. 2002b. Cerebellar function in consolidation of a motor memory. *Neuron,* 34**,** 1011-20.

ATTWELL, P. J., RAHMAN, S., IVARSSON, M. & YEO, C. H. 1999. Cerebellar cortical AMPA-kainate receptor blockade prevents performance of classically conditioned nictitating membrane responses. *J Neurosci,* 19**,** RC45.

ATTWELL, P. J., RAHMAN, S. & YEO, C. H. 2001. Acquisition of eyeblink conditioning is critically dependent on normal function in cerebellar cortical lobule HVI. *J Neurosci,* 21**,** 5715-22.

BARILI, P., BRONZETTI, E., RICCI, A., ZACCHEO, D. & AMENTA, F. 2000. Microanatomical localization of dopamine receptor protein immunoreactivity in the rat cerebellar cortex. *Brain Res,* 854**,** 130-138.

BARMACK, N. H., BAUGHMAN, R. W. & ECKENSTEIN, F. P. 1992a. Cholinergic innervation of the cerebellum of rat, rabbit, cat, and monkey as revealed by choline acetyltransferase activity and immunohistochemistry. *J Comp Neurol,* 317**,** 233-249.

BARMACK, N. H., BAUGHMAN, R. W., ECKENSTEIN, F. P. & SHOJAKU, H. 1992b. Secondary vestibular cholinergic projection to the cerebellum of rabbit and rat as revealed by choline acetyltransferase immunohistochemistry, retrograde and orthograde tracers. *J Comp Neurol,* 317**,** 250-270.

BARNES, N. M. & SHARP, T. 1999. A review of central 5-HT receptors and their function. *Neuropharmacology,* 38**,** 1083-152.

BARROS, D. M., MELLO E SOUZA, T., DE DAVID, T., CHOI, H., AGUZZOLI, A., MADCHE, C., ARDENGHI, P., MEDINA, J. H. & IZQUIERDO, I. 2001. Simultaneous modulation of retrieval by dopaminergic D1, β-noradrenergic, serotonergic-1A and cholinergic muscarinic receptors in cortical structures of the rat. *Beh Brain Res,* 124**,** 1-7.

BEAUDET, A. & DESCARRIES, L. 1978. The monoamine innervation of rat cerebral cortex: Synaptic and nonsynaptic axon terminals. *Neuroscience,* 3**,** 851-860.

BEAUDET, A. & SOTELO, C. 1981. Synaptic remodeling of serotonin axon terminals in rat agranular cerebellum. *Brain Res,* 206**,** 305-329.

BEIERLEIN, M. & REGEHR, W. G. 2006. Brief Bursts of Parallel Fiber Activity Trigger Calcium Signals in Bergmann Glia. *J Neurosci,* 26**,** 6958-6967.

BELCHEVA, I., BELCHEVA, S., PETKOV, V. V., HADJIIVANOVA, C. & PETKOV, V. D. 1997. Behavorial responses to the 5-HT1A receptor antagonist NAN190 injected into rat CA1 hippocampal area. *General Pharmacology: The Vascular System,* 28**,** 435-441.

BELL, C. C. 2002. Evolution of cerebellum-like structures. *Brain Behav Evol,* 59**,** 312-26.

BELMEGUENAI, A. & HANSEL, C. 2005. A Role for Protein Phosphatases 1, 2A, and 2B in Cerebellar Long-Term Potentiation. *J Neurosci,* 25**,** 10768-10772.

BENGTSSON, F. & HESSLOW, G. 2006. Cerebellar control of the inferior olive. *Cerebellum,* 5**,** 7-14.

BENGTSSON, F., SVENSSON, P. & HESSLOW, G. 2004. Feedback control of Purkinje cell activity by the cerebello-olivary pathway. *Eur J Neurosci,* 20**,** 2999-3005.

BERGLES, D. E., DZUBAY, J. A. & JAHR, C. E. 1997. Glutamate transporter currents in Bergmann glial cells follow the time course of extrasynaptic glutamate. *Proc Natl Acad Sci U S A,* 94**,** 14821-14825.

BERNARDINELLI, Y., MULLER, D. & NIKONENKO, I. 2014. Astrocyte-Synapse Structural Plasticity. *Neural Plast,* 2014**,** 232105.

BERRIDGE, C. W. & WATERHOUSE, B. D. 2003. The locus coeruleus-noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. *Brain Res Brain Res Rev,* 42**,** 33-84.

BICKFORD, P. 1993. Motor learning deficits in aged rats are correlated with loss of cerebellar noradrenergic function. *Brain Res,* 620**,** 133-138.

BISHOP, G. A. & HO, R. H. 1985. The distribution and origin of serotonin immunoreactivity in the rat cerebellum. *Brain Res,* 331**,** 195-207.

BLOEDEL, J. R. & BRACHA, V. 1995. On the cerebellum, cutaneomuscular reflexes, movement control and the elusive engrams of memory. *Beh Brain Res,* 68**,** 1-44.

BLOOM, F. E., HOFFER, B. J. & SIGGINS, G. R. 1971. Studies on norepinephrine-containing afferents to Purkinje cells of art cerebellum. I. Localization of the fibers and their synapses. *Brain Res,* 25**,** 501-21.

BOLK, L. 1906. *Das Cerebellum der Saugetiere,* Jena, Germany, Fischer.

BOOZE, R. M., CRISOSTOMO, E. A. & DAVIS, J. N. 1989. Species differences in the localization and number of CNS beta adrenergic receptors: rat versus guinea pig. *J Pharmacol Exp Ther,* 249**,** 911-20.

BOSCHERT, U., AMARA, D. A., SEGU, L. & HEN, R. 1994. The mouse 5-hydroxytryptamine1B receptor is localized predominantly on axon terminals. *Neuroscience,* 58**,** 167-82.

BOURET, S. & SARA, S. J. 2005. Network reset: a simplified overarching theory of locus coeruleus noradrenaline function. *Trends Neurosci,* 28**,** 574-82.

BOURET, S. & RICHMOND, B. J. 2009. Relation of locus coeruleus neurons in monkeys to Pavlovian and operant behaviors. *J Neurophysiol,* 101**,** 898-911.

BOYDEN, E. S., KATOH, A. & RAYMOND, J. L. 2004. Cerebellum-dependent learning: the role of multiple plasticity mechanisms. *Annu Rev Neurosci,* 27**,** 581-609.

BRACHA, V. 2004. Role of the cerebellum in eyeblink conditioning. *Prog Brain Res,* 143**,** 331-9.

BRAITENBERG, V. & ATWOOD, R. P. 1958. Morphological observations on the cerebellar cortex. *J Comp Neurol,* 109**,** 1-33.

BROCHU, G., MALER, L. & HAWKES, R. 1990. Zebrin II: a polypeptide antigen expressed selectively by Purkinje cells reveals compartments in rat and fish cerebellum. *J Comp Neurol,* 291**,** 538-52.

BUISSERET-DELMAS, C. & ANGAUT, P. 1993. The cerebellar olivo-corticonuclear connections in the rat. *Prog Neurobiol,* 40**,** 63-87.

BURHANS, L. B. & SCHREURS, B. G. 2013. Inactivation of the central nucleus of the amygdala blocks classical conditioning but not conditioning-specific reflex modification of rabbit heart rate. *Neurobiol Learn Mem,* 100**,** 88-97.

CAREY, M. R. & REGEHR, W. G. 2009. Noradrenergic control of associative synaptic plasticity by selective modulation of instructive signals. *Neuron,* 62**,** 112-22.

CARLI, M. & SAMANIN, R. 1992. 8-Hydroxy-2-(di-n-propylamino)tetralin impairs spatial learning in a water maze: role of postsynaptic 5-HT1A receptors. *Br J Pharmacol,* 105**,** 720-726.

CARLI, M., TRANCHINA, S. & SAMANIN, R. 1992. 8-Hydroxy-2-(di-n-propylamino)tetralin, a 5-HT1A receptor agonist, impairs performance in a passive avoidance task. *European Journal of Pharmacology,* 211**,** 227-234.

CARLI, M., TATARCZYNSKA, E., CERVO, L. & SAMANIN, R. 1993. Stimulation of hippocampal 5-HT1A receptors causes amnesia and anxiolytic-like but not antidepressant-like effects in the rat. *Eur J Pharmacol,* 234**,** 215-221.

CARLI, M., LUSCHI, R. & SAMANIN, R. 1996. (S)-WAY 100135, a 5-HT1A receptor antagonist, prevents the impairment of spatial learning caused by intrahippocampal scopolamine. *Eur J Neurosci,* 283**,** 133-9.

CARLI, M., SILVA, S., BALDUCCI, C. & SAMANIN, R. 1999. WAY 100635, a 5-HT1A receptor antagonist, prevents the impairment of spatial learning caused by blockade of hippocampal NMDA receptors. *Neuropharmacology,* 38**,** 1165-1173.

CARTFORD, M. C., ALLGEIER, C. A. & BICKFORD, P. C. 2002. The Effects of β-Noradrenergic Receptor Blockade on Acquisition of Eyeblink Conditioning in 3-Month-Old F344 Rats. *Neurobiol Learn Mem,* 78**,** 246-257.

CARTFORD, M. C., SAMEC, A., FISTER, M. & BICKFORD, P. C. 2004a. Cerebellar norepinephrine modulates learning of delay classical eyeblink conditioning: evidence for post-synaptic signaling via PKA. *Learn Mem,* 11**,** 732-7.

CARTFORD, M. C., GOULD, T. & BICKFORD, P. C. 2004b. A central role for norepinephrine in the modulation of cerebellar learning tasks. *Behav Cogn Neurosci Rev,* 3**,** 131-8.

CASTEJON, O. J., DAILEY, M., APKARIAN, R. P. & CASTEJON, H. V. 2002. Correlative Microscopy of Cerebellar Bergmann Glial Cells. *Microscopy and Microanalysis,* 8**,** 1032-1033.

CERMINARA, N. L. & RAWSON, J. A. 2004. Evidence that Climbing Fibers Control an Intrinsic Spike Generator in Cerebellar Purkinje Cells. *J Neurosci,* 24**,** 4510-4517.

CHAMBERS, W. W. & SPRAGUE, J. M. 1955a. Functional localization in the cerebellum. I. Organization in longitudinal cortico-nuclear zones and their contribution to the control of posture, both extrapyramidal and pyramidal. *J Comp Neurol,* 103**,** 105-29.

CHAMBERS, W. W. & SPRAGUE, J. M. 1955b. Functional localization in the cerebellum. II. Somatotopic organization in cortex and nuclei. *AMA Arch Neurol Psychiatry,* 74**,** 653-80.

CHAN-PALAY, V. 1973. On the identification of the afferent axon terminals in the nucleus lateralis of the cerebellum an electron microscope study. *Zeitschrift für Anatomie und Entwicklungsgeschichte,* 142**,** 149-186.

CHAN-PALAY, V. 1975. Fine structure of labelled axons in the cerebellar cortex and nuclei of rodents and primates after intraventricular infusions with tritiated serotonin. *Anat Embryol (Berl),* 148**,** 235-65.

CHAN-PALAY, V. 1977. The Cerebellar Dentate Nucleus. *Cerebellar Dentate Nucleus: Organization, Cytology and Transmitters.* Berlin, Heidelberg: Springer Berlin Heidelberg.

CHANDLER, D. J., LAMPERSKI, C. S. & WATERHOUSE, B. D. 2013. Identification and distribution of projections from monoaminergic and cholinergic nuclei to functionally differentiated subregions of prefrontal cortex. *Brain Res,* 1522**,** 38-58.

CHANDLER, D. J., GAO, W. J. & WATERHOUSE, B. D. 2014. Heterogeneous organization of the locus coeruleus projections to prefrontal and motor cortices. *Proc Natl Acad Sci U S A,* 111**,** 6816-21.

CHEUN, J. E. & YEH, H. H. 1992. Modulation of GABAA receptor-activated current by norepinephrine in cerebellar Purkinje cells. *Neuroscience,* 51**,** 951-60.

CHOI, D.-S. & MAROTEAUX, L. 1996. Immunohistochemical localisation of the serotonin 5-HT2B receptor in mouse gut, cardiovascular system, and brain. *FEBS Letters,* 391**,** 45-51.

CHU, N.-S. & BLOOM, F. E. 1974. The catecholamine-containing neurons in the cat dorsolateral pontine tegmentum: Distribution of the cell bodies and some axonal projections. *Brain Res,* 66**,** 1-21.

CLARK, G. A., MCCORMICK, D. A., LAVOND, D. G. & THOMPSON, R. F. 1984. Effects of lesions of cerebellar nuclei on conditioned behavioral and hippocampal neuronal responses. *Brain Res,* 291**,** 125-36.

COOKE, S. F., ATTWELL, P. J. & YEO, C. H. 2004. Temporal properties of cerebellar-dependent memory consolidation. *J Neurosci,* 24**,** 2934-41.

CORBETTA, M., PATEL, G. & SHULMAN, G. L. 2008. The Reorienting System of the Human Brain: From Environment to Theory of Mind. *Neuron,* 58**,** 306-324.

CORNEA-HEBERT, V., RIAD, M., WU, C., SINGH, S. K. & DESCARRIES, L. 1999. Cellular and subcellular distribution of the serotonin 5-HT2A receptor in the central nervous system of adult rat. *J Comp Neurol,* 409**,** 187-209.

CORTEEN, N. L., COLE, T. M., SARNA, A., SIEGHART, W. & SWINNY, J. D. 2011. Localization of GABA-A receptor alpha subunits on neurochemically distinct cell types in the rat locus coeruleus. *Eur J Neurosci,* 34**,** 250-62.

COUTINHO, V., MUTOH, H. & KNOPFEL, T. 2004. Functional topology of the mossy fibre-granule cell--Purkinje cell system revealed by imaging of intrinsic fluorescence in mouse cerebellum. *Eur J Neurosci,* 20**,** 740-8.

CREPEL, F., DEBONO, M. & FLORES, R. 1987. Alpha-adrenergic inhibition of rat cerebellar Purkinje cells in vitro: a voltage-clamp study. *J Physiol,* 383**,** 487-498.

D'ANGELO, E. & DE ZEEUW, C. I. 2009. Timing and plasticity in the cerebellum: focus on the granular layer. *Trends Neurosci,* 32**,** 30-40.

D'ANGELO, E., ROSSI, P., ARMANO, S. & TAGLIETTI, V. 1999. Evidence for NMDA and mGlu Receptor-Dependent Long-Term Potentiation of Mossy Fiber–Granule Cell Transmission in Rat Cerebellum. *J Neurophysiol,* 81**,** 277-287.

D'ANGELO, E., SOLINAS, S., MAPELLI, J., GANDOLFI, D., MAPELLI, L. & PRESTORI, F. 2013. The cerebellar Golgi cell and spatiotemporal organization of granular layer activity. *Frontiers in Neural Circuits,* 7**,** 93.

DARROW, E. J., STRAHLENDORF, H. K. & STRAHLENDORF, J. C. 1990. Response of cerebellar Purkinje cells to serotonin and the 5-HT1A agonists 8-OH-DPAT and ipsapirone in vitro. *Eur J Pharmacol,* 175**,** 145-53.

DAY, H. E., CAMPEAU, S., WATSON, S. J., JR. & AKIL, H. 1997. Distribution of alpha 1a-, alpha 1b- and alpha 1d-adrenergic receptor mRNA in the rat brain and spinal cord. *J Chem Neuroanat,* 13**,** 115-39.

DE BLAS, A. L. 1984. Monoclonal antibodies to specific astroglial and neuronal antigens reveal the cytoarchitecture of the Bergmann glia fibres in the cerebellum. *J Neurosci,* 4**,** 265-73.

DE ZEEUW, C. I. & YEO, C. H. 2005. Time and tide in cerebellar memory formation. *Curr Opin Neurobiol,* 15**,** 667-74.

DE ZEEUW, C. I., HANSEL, C., BIAN, F., KOEKKOEK, S. K., VAN ALPHEN, A. M., LINDEN, D. J. & OBERDICK, J. 1998. Expression of a protein kinase C inhibitor in Purkinje cells blocks cerebellar LTD and adaptation of the vestibulo-ocular reflex. *Neuron,* 20**,** 495-508.

DE ZEEUW, C. I., HOEBEEK, F. E., BOSMAN, L. W., SCHONEWILLE, M., WITTER, L. & KOEKKOEK, S. K. 2011. Spatiotemporal firing patterns in the cerebellum. *Nat Rev Neurosci,* 12**,** 327-44.

DE ZEEUW, C. I. & HOOGLAND, T. M. 2015. Reappraisal of Bergmann glial cells as modulators of cerebellar circuit function. *Frontiers in Cellular Neuroscience,* 9.

DEAN, I., ROBERTSON, S. J. & EDWARDS, F. A. 2003. Serotonin drives a novel GABAergic synaptic current recorded in rat cerebellar purkinje cells: a Lugaro cell to Purkinje cell synapse. *J Neurosci,* 23**,** 4457-69.

DEAN, P. & PORRILL, J. 2014. Decorrelation Learning in the Cerebellum: Computational Analysis and Experimental Questions. *Prog Brain Res,* 210*,* 157-192.

DEAN, P., PORRILL, J. & STONE, J. V. 2004. Visual awareness and the cerebellum: possible role of decorrelation control. *Prog Brain Res,* 144, 61-75

DEAN, P., PORRILL, J., EKEROT, C.-F. & JORNTELL, H. 2010. The cerebellar microcircuit as an adaptive filter: experimental and computational evidence. *Nat Rev Neurosci,* 11**,** 30-43.

DEAN, P., ANDERSON, S., PORRILL, J. & JÖRNTELL, H. 2013. An adaptive filter model of cerebellar zone C3 as a basis for safe limb control? *J Physiol,* 591**,** 5459-5474.

DEROUICHE, A. & FROTSCHER, M. 1991. Astroglial processes around identified glutamatergic synapses contain glutamine synthetase: evidence for transmitter degradation. *Brain Res,* 552**,** 346-350.

DESCARRIES, L. & MECHAWAR, N. 2000. Ultrastructural evidence for diffuse transmission by monoamine and acetylcholine neurons of the central nervous system. *Prog Brain Res,* 125. 27-47

DESMOND, J. E. & MOORE, J. W. 1982. A brain stem region essential for the classically conditioned but not unconditioned nictitating membrane response. *Physiol Behav,* 28**,** 1029-33.

DI MAURO, M., FRETTO, G., CALDERA, M., LI VOLSI, G., LICATA, F., CIRANNA, L. & SANTANGELO, F. 2003. Noradrenaline and 5-hydroxytryptamine in cerebellar nuclei of the rat: functional effects on neuronal firing. *Neurosci Lett,* 347**,** 101-5.

DI MAURO, M., LI VOLSI, G. & LICATA, F. 2013. Noradrenergic control of neuronal firing in cerebellar nuclei: modulation of GABA responses. *Cerebellum,* 12**,** 350-61.

DIETRICHS, E. & WALBERG, F. 1979. The cerebellar corticonuclear and nucleocortical projections in the cat as studied with anterograde and retrograde transport of horseradish peroxidase. I. The paramedian lobule. *Anat Embryol (Berl),* 158**,** 13-39.

DIEUDONNÉ, S. 2001. Serotonergic neuromodulation in the cerebellar cortex: cellular, synaptic, and molecular basis. *Neuroscientist,* 7**,** 207-19.

DIEUDONNE, S. & DUMOULIN, A. 2000. Serotonin-driven long-range inhibitory connections in the cerebellar cortex. *J Neurosci,* 20**,** 1837-48.

DIÑO, M. R., WILLARD, F. H. & MUGNAINI, E. 1999. Distribution of unipolar brush cells and other calretinin immunoreactive components in the mammalian cerebellar cortex. *J Neurocytol,* 28**,** 99-123.

DIÑO, M. R., SCHUERGER, R. J., LIU, Y. B., SLATER, N. T. & MUGNAINI, E. 2000. Unipolar brush cell: a potential feedforward excitatory interneuron of the cerebellum. *Neuroscience,* 98**,** 625-636.

DINOPOULOS, A., DORI, I. & PARNAVELAS, J. G. 1995. Serotonergic innervation of the lateral geniculate nucleus of the rat during postnatal development: A light and electron microscopic immunocytochemical analysis. *J Comp Neurol,* 363**,** 532-544.

DOW, R. S. & MORUZZI, G. 1958. *The physiology and pathology of the cerebellum,* Minneapolis, University of Minnesota Press.

DUMOULIN, A., TRILLER, A. & DIEUDONNÉ, S. 2001. IPSC kinetics at identified GABAergic and mixed GABAergic and glycinergic synapses onto cerebellar Golgi cells. *J Neurosci,* 21**,** 6045-57.

DUXON, M. S., FLANIGAN, T. P., REAVLEY, A. C., BAXTER, G. S., BLACKBURN, T. P. & FONE, K. C. 1997. Evidence for expression of the 5-hydroxytryptamine-2B receptor protein in the rat central nervous system. *Neuroscience,* 76**,** 323-9.

ECCLES, J. C., ITO, M. & SZENTÁGOTHAI, J. N. 1967. *The cerebellum as a neuronal machine,* Berlin, New York etc., Springer-Verlag.

ECCLES, J. C., LLINAS, R. & SASAKI, K. 1966. The mossy fibre-granule cell relay of the cerebellum and its inhibitory control by Golgi cells. *Exp Brain Res,* 1**,** 82-101.

EGASHIRA, N., YANO, A., ISHIGAMI, N., MISHIMA, K., IWASAKI, K., FUJIOKA, M., MATSUSHITA, M., NISHIMURA, R. & FUJIWARA, M. 2006. Investigation of mechanisms mediating 8-OH-DPAT-induced impairment of spatial memory: Involvement of 5-HT1A receptors in the dorsal hippocampus in rats. *Brain Res,* 1069**,** 54-62.

EKEROT, C. F. & LARSON, B. 1982. Branching of olivary axons to innervate pairs of sagittal zones in the cerebellar anterior lobe of the cat. *Exp Brain Res,* 48**,** 185-98.

EKEROT, C. F. & KANO, M. 1985. Long-term depression of parallel fibre synapses following stimulation of climbing fibres. *Brain Res,* 342**,** 357-360.

EKEROT, C. F. & JORNTELL, H. 2001. Parallel fibre receptive fields of Purkinje cells and interneurons are climbing fibre-specific. *Eur J Neurosci,* 13**,** 1303-10.

FEIL, R., HARTMANN, J., LUO, C., WOLFSGRUBER, W., SCHILLING, K., FEIL, S., BARSKI, J. J., MEYER, M., KONNERTH, A., DE ZEEUW, C. I. & HOFMANN, F. 2003. Impairment of LTD and cerebellar learning by Purkinje cell–specific ablation of cGMP-dependent protein kinase I. *The Journal of Cell Biology,* 163**,** 295-302.

FELTEN, D. L., FELTEN, S. Y., PERRY, K. W., FULLER, R. W., NURNBERGER, J. I. & GHETTI, B. 1986. Noradrenergic innervation of the cerebellar cortex in normal and in Purkinje cell degeneration mutant mice: evidence for long term survival following loss of the two major cerebellar cortical neuronal populations. *Neuroscience,* 18**,** 783-93.

FINE, E. J., IONITA, C. C. & LOHR, L. 2002. The history of the development of the cerebellar examination. *Semin Neurol,* 22**,** 375-84.

FLOOD, J. F. & CHERKIN, A. 1987. Fluoxetine enhances memory processing in mice. *Psychopharmacology (Berl),* 93**,** 36-43.

FOOTE, S. L., ASTON-JONES, G. & BLOOM, F. E. 1980. Impulse activity of locus coeruleus neurons in awake rats and monkeys is a function of sensory stimulation and arousal. *Proc Natl Acad Sci U S A,* 77**,** 3033-7.

FOOTE, S. L., FREEDMAN, R. & OLIVER, A. P. 1975. Effects of putative neurotransmitters on neuronal activity in monkey auditory cortex. *Brain Res,* 86**,** 229-242.

FOX, C. A., HILLMAN, D. E., SIEGESMUND, K. A. & DUTTA, C. R. 1967. The Primate Cerebellar Cortex: A Golgi and Electron Microscopic Study. *Prog Brain Res,* 25, 174-225

FREDETTE, B. J. & MUGNAINI, E. 1991. The GABAergic cerebello-olivary projection in the rat. *Anat Embryol (Berl),* 184**,** 225-43.

FREEDMAN, R., HOFFER, B. J., PURO, D. & WOODWARD, D. J. 1976. Noradrenaline modulation of the responses of the cerebellar Purkinje cell to afferent synaptic activity. *Br J Pharmacol,* 57**,** 603-5.

FREEDMAN, R., HOFFER, B. J., WOODWARD, D. J. & PURO, D. 1977. Interaction of norepinephrine with cerebellar activity evoked by mossy and climbing fibers. *Exp Neurol,* 55**,** 269-88.

FREY, U., HUANG, Y. & KANDEL, E. 1993. Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. *Science,* 260**,** 1661-1664.

FUJITA, M. 1982. Adaptive filter model of the cerebellum. *Biol Cybern,* 45**,** 195-206.

GALLAGHER, M., KAPP, B., MUSTY, R. & DRISCOLL, P. 1977. Memory formation: evidence for a specific neurochemical system in the amygdala. *Science,* 198**,** 423-425.

GAO, W., CHEN, G., REINERT, K. C. & EBNER, T. J. 2006. Cerebellar cortical molecular layer inhibition is organized in parasagittal zones. *J Neurosci,* 26**,** 8377-87.

GARCIA, K. S. & MAUK, M. D. 1998. Pharmacological analysis of cerebellar contributions to the timing and expression of conditioned eyelid responses. *Neuropharmacology,* 37**,** 471-480.

GARCIA, K. S., STEELE, P. M. & MAUK, M. D. 1999. Cerebellar cortex lesions prevent acquisition of conditioned eyelid responses. *J Neurosci,* 19**,** 10940-7.

GARWICZ, M. & EKEROT, C. F. 1994. Topographical organization of the cerebellar cortical projection to nucleus interpositus anterior in the cat. *J Physiol,* 474**,** 245-60.

GÉRARD, C., MARTRES, M.-P., LEFÈVRE, K., MIQUEL, M.-C., VERGÉ, D., LANFUMEY, L., DOUCET, E., HAMON, M. & EL MESTIKAWY, S. 1997. Immuno-localization of serotonin 5-HT6 receptor-like material in the rat central nervous system. *Brain Res,* 746**,** 207-219.

GERRITS, N. M. & VOOGD, J. 1987. The projection of the nucleus reticularis tegmenti pontis and adjacent regions of the pontine nuclei to the central cerebellar nuclei in the cat. *J Comp Neurol,* 258**,** 52-62.

GEURTS, F. J., TIMMERMANS, J., SHIGEMOTO, R. & DE SCHUTTER, E. 2001. Morphological and neurochemical differentiation of large granular layer interneurons in the adult rat cerebellum. *Neuroscience,* 104**,** 499-512.

GEURTS, F. J., DE SCHUTTER, E. & TIMMERMANS, J. P. 2002. Localization of 5-HT2A, 5-HT3, 5-HT5A and 5-HT7 receptor-like immunoreactivity in the rat cerebellum. *J Chem Neuroanat,* 24**,** 65-74.

GIANLORENÇO, A. C. L., CANTO-DE-SOUZA, A. & MATTIOLI, R. 2011. Microinjection of histamine into the cerebellar vermis impairs emotional memory consolidation in mice. *Brain Res Bull,* 86**,** 134-138.

GIANLORENÇO, A. C. L., SERAFIM, K. R., CANTO-DE-SOUZA, A. & MATTIOLI, R. 2012. Emotional memory consolidation impairment induced by histamine is mediated by H1 but not H2 receptors. *Brain Res Bull,* 89**,** 197-202.

GIANLORENÇO, A. C. L., CANTO-DE-SOUZA, A. & MATTIOLI, R. 2013. Intra-cerebellar microinjection of histamine enhances memory consolidation of inhibitory avoidance learning in mice via H2 receptors. *Neurosci Lett,* 557, Part B**,** 159-164.

GIANLORENÇO, A. C. L., RIBOLDI, A. M., SILVA-MARQUES, B. & MATTIOLI, R. 2015. Cerebellar vermis H2 receptors mediate fear memory consolidation in mice. *Neurosci Lett,* 587**,** 57-61.

GIBBS, M. E. & NG, K. T. 1976. Memory formation: A new three-phase model. *Neurosci Lett,* 2**,** 165-169.

GIBBS, M. E. & SUMMERS, R. J. 2002. Role of adrenoceptor subtypes in memory consolidation. *Prog Neurobiol,* 67**,** 345-91.

GILBERT, P. F. 1974. A theory of memory that explains the function and structure of the cerebellum. *Brain Res,* 70**,** 1-18.

GILBERT, P. F. 1975. How the cerebellum could memorise movements. *Nature,* 254**,** 688-689.

GLICKSTEIN, M. & DORON, K. 2008. Cerebellum: connections and functions. *Cerebellum,* 7**,** 589-94.

GLICKSTEIN, M., SULTAN, F. & VOOGD, J. 2011. Functional localization in the cerebellum. *Cortex,* 47**,** 59-80.

GOLD, P. E. & MCGAUGH, J. L. 1975. A single-trace, two process view of memory storage processes. *In:* DEUTSCH, J. & DEUTSCH, D. (eds.) *Short-term memory.* New York: Academic Press.

GONZALEZ-JOEKES, J. & SCHREURS, B. G. 2012. Anatomical characterization of a rabbit cerebellar eyeblink premotor pathway using pseudorabies and identification of a local modulatory network in anterior interpositus. *J Neurosci,* 32**,** 12472-87.

GORMEZANO, I., SCHNEIDERMAN, N., DEAUX, E. & FUENTES, I. 1962. Nictitating membrane: classical conditioning and extinction in the albino rabbit. *Science,* 138**,** 33-4.

GOULD, T. J. 1998. Beta-adrenergic involvement in acquisition vs. extinction of a classically conditioned eye blink response in rabbits. *Brain Res,* 780**,** 174-7.

GOULD, T. J. & BICKFORD, P. C. 1997. Age-Related Deficits in the Cerebellar BetaAdrenergic Signal Transduction Cascade in Fischer 344 Rats. *J Pharmacol Expl Ther,* 281**,** 965-971.

GOULD, T. J., ADAMS, C. E. & BICKFORD, P. C. 1997. β-Adrenergic Modulation of GABAergic Inhibition in the Deep Cerebellar Nuclei of F344 Rats. *Neuropharmacology,* 36**,** 75-81.

GREEN, J. T. & STEINMETZ, J. E. 2005. Purkinje cell activity in the cerebellar anterior lobe after rabbit eyeblink conditioning. *Learn Mem,* 12**,** 260-9.

GROENEWEGEN, H. J. & VOOGD, J. 1977. The parasagittal zonation within the olivocerebellar projection. I. Climbing fiber distribution in the vermis of cat cerebellum. *J Comp Neurol,* 174**,** 417-88.

GROENEWEGEN, H. J., VOOGD, J. & FREEDMAN, S. L. 1979. The parasagittal zonation within the olivocerebellar projection. II. Climbing fiber distribution in the

intermediate and hemispheric parts of cat cerebellum. *J Comp Neurol,* 183**,** 551-601.

GROSCHE, J., MATYASH, V., MOLLER, T., VERKHRATSKY, A., REICHENBACH, A. & KETTENMANN, H. 1999. Microdomains for neuron-glia interaction: parallel fiber signaling to Bergmann glial cells. *Nat Neurosci,* 2**,** 139-143.

GROSCHE, J., KETTENMANN, H. & REICHENBACH, A. 2002. Bergmann glial cells form distinct morphological structures to interact with cerebellar neurons. *J Neurosci Res,* 68**,** 138-149.

GRUART, A., PASTOR, A. M., ARMENGOL, J. A. & DELGADO-GARCIA, J. M. 1997. Involvement of cerebellar cortex and nuclei in the genesis and control of unconditioned and conditioned eyelid motor responses. *Prog Brain Res,* 114**,** 511-28.

HALVERSON, H. E., KHILKEVICH, A. & MAUK, M. D. 2015. Relating Cerebellar Purkinje Cell Activity to the Timing and Amplitude of Conditioned Eyelid Responses. *J Neurosci,* 35**,** 7813-7832.

HANSEL, C., LINDEN, D. J. & D'ANGELO, E. 2001. Beyond parallel fiber LTD: the diversity of synaptic and non-synaptic plasticity in the cerebellum. *Nat Neurosci,* 4**,** 467-75.

HARDIMAN, M. J., RAMNANI, N. & YEO, C. H. 1996. Reversible inactivations of the cerebellum with muscimol prevent the acquisition and extinction of conditioned nictitating membrane responses in the rabbit. *Exp Brain Res,* 110**,** 235-47.

HARLEY, C. W. 2004. Norepinephrine and dopamine as learning signals. *Neural Plast,* 11**,** 191-204.

HARLEY, C. W. & MILWAY, J. S. 1986. Glutamate ejection in the locus coeruleus enhances the perforant path-evoked population spike in the dentate gyrus. *Exp Brain Res,* 63**,** 143-50.

HARVEY, J. A. 2003. Role of the serotonin 5-HT(2A) receptor in learning. *Learn Mem,* 10**,** 355-62.

HARVEY, R. J. & NAPPER, R. M. 1988. Quantitative study of granule and Purkinje cells in the cerebellar cortex of the rat. *J Comp Neurol,* 274**,** 151-7.

HARVEY, R. J. & NAPPER, R. M. 1991. Quantitative studies on the mammalian cerebellum. *Prog Neurobiol,* 36**,** 437-63.

HARVEY, J. A., GORMEZANO, I., COOL, H. V. & SCHINDLER, C. W. 1988. Effects of LSD on classical conditioning as a function of CS-UCS interval: relationship to reflex facilitation. *Pharmacol Biochem Behav.,* 30**,** 433-441.

HAUSSER, M. & CLARK, B. A. 1997. Tonic synaptic inhibition modulates neuronal output pattern and spatiotemporal synaptic integration. *Neuron,* 19**,** 665-78.

HAVEKES, R., CANTON, D. A., PARK, A. J., HUANG, T., NIE, T., DAY, J. P., GUERCIO, L. A., GRIMES, Q., LUCZAK, V., GELMAN, I. H., BAILLIE, G. S., SCOTT, J. D. & ABEL, T. 2012. Gravin orchestrates PKA and β_2-adrenergic receptor signaling critical for synaptic plasticity and memory. *J Neurosci,* 32**,** 18137-18149.

HEINEY, S. A., KIM, J., AUGUSTINE, G. J. & MEDINA, J. F. 2014. Precise Control of Movement Kinematics by Optogenetic Inhibition of Purkinje Cell Activity. *J Neurosci,* 34**,** 2321-2330.

HEROLD, S., HECKER, C., DEITMER, J. W. & BROCKHAUS, J. 2005. alpha1-Adrenergic modulation of synaptic input to Purkinje neurons in rat cerebellar brain slices. *J Neurosci Res,* 82**,** 571-9.

HERON, C., GOULD, T. J. & BICKFORD, P. 1996. Acquisition of a runway motor learning task is impaired by a beta adrenergic antagonist in F344 rats. *Beh Brain Res,* 78**,** 235-241.

HERRERO, L., PARDOE, J. & APPS, R. 2002. Pontine and lateral reticular projections to the c1 zone in lobulus simplex and paramedian lobule of the rat cerebellar cortex. *Cerebellum,* 1**,** 185-99.

HESSLOW, G. 1986. Inhibition of inferior olivary transmission by mesencephalic stimulation in the cat. *Neurosci Lett,* 63**,** 76-80.

HESSLOW, G. 1994a. Correspondence between climbing fibre input and motor output in eyeblink-related areas in cat cerebellar cortex. *J Physiol,* 476**,** 229-44.

HESSLOW, G. 1994b. Inhibition of classically conditioned eyeblink responses by stimulation of the cerebellar cortex in the decerebrate cat. *J Physiol,* 476**,** 245-56.

HESSLOW, G., SVENSSON, P. & IVARSSON, M. 1999. Learned movements elicited by direct stimulation of cerebellar mossy fiber afferents. *Neuron,* 24**,** 179-85.

HESSLOW, G. & YEO, C. H. 2002. The Functional Anatomy of Skeletal Conditioning. *In:* MOORE, J. W. (ed.) *A Neuroscientist's Guide to Classical Conditioning.* New York: Springer.

HESSLOW, G., JIRENHED, D. A., RASMUSSEN, A. & JOHANSSON, F. 2013. Classical conditioning of motor responses: What is the learning mechanism? *Neural Networks,* 47**,** 81-87.

HICKS, T. P., KRUPA, M. & CREPEL, F. 1989. Selective effects of serotonin upon excitatory amino acid-induced depolarizations of Purkinje cells in cerebellar slices from young rats. *Brain Res,* 492**,** 371-6.

HIRONO, M. & OBATA, K. 2006. Alpha-adrenoceptive dual modulation of inhibitory GABAergic inputs to Purkinje cells in the mouse cerebellum. *J Neurophysiol,* 95**,** 700-8.

HIRONO, M., MATSUNAGA, W., CHIMURA, T. & OBATA, K. 2008. Developmental enhancement of alpha2-adrenoceptor-mediated suppression of inhibitory synaptic transmission onto mouse cerebellar Purkinje cells. *Neuroscience,* 156**,** 143-54.

HOFFER, B. J., SIGGINS, G. R. & BLOOM, F. E. 1971. Studies on norepinephrine-containing afferents to Purkinje cells of rat cerebellum. II. Sensitivity of Purkinje cells to norepinephrine and related substances administered by microiontophoresis. *Brain Res,* 25**,** 523-34.

HOFFER, B. J., SIGGINS, G. R., OLIVER, A. P. & BLOOM, F. E. 1973. Activation of the pathway from locus coeruleus to rat cerebellar Purkinje neurons: pharmacological evidence of noradrenergic central inhibition. *J Pharmacol Exp Ther,* 184**,** 553-69.

HÖKFELT, T. & FUXE, K. 1969. Cerebellar monoamine nerve terminals, a new type of afferent fibers to the cortex cerebelli. *Exp Brain Res,* 9**,** 63-72.

HOLMES, G. 1939. The Cerebellum of Man. *Brain,* 62**,** 30.

HOUK, J. C., BUCKINGHAM, J. T. & BARTO, A. G. 1996. Models of the cerebellum and motor learning. *Beh Brain Sci,* 19**,** 16.

HUANG, Y.-Y. & KANDEL, E. R. 1998. Postsynaptic Induction and PKA-Dependent Expression of LTP in the Lateral Amygdala. *Neuron,* 21**,** 169-178.

HUANG, C. M., WANG, L. & HUANG, R. H. 2006. Cerebellar granule cell: ascending axon and parallel fiber. *Eur J Neurosci,* 23**,** 1731-7.

IKAI, Y., TAKADA, M., SHINONAGA, Y. & MIZUNO, N. 1992. Dopaminergic and non-dopaminergic neurons in the ventral tegmental area of the rat project, respectively, to the cerebellar cortex and deep cerebellar nuclei. *Neuroscience,* 51**,** 719-728.

INTROINI-COLLISON, I. B., NAGAHARA, A. H. & MCGAUGH, J. L. 1989. Memory enhancement with intra-amygdala post-training naloxone is blocked by concurrent administration of propranolol. *Brain Res,* 476**,** 94-101.

ISOPE, P. & BARBOUR, B. 2002. Properties of unitary granule cell-->Purkinje cell synapses in adult rat cerebellar slices. *J Neurosci,* 22**,** 9668-78.

ITO, M. 1972. Neural design of the cerebellar motor control system. *Brain Res,* 40**,** 81-84.

ITO, M. 1982. Cerebellar control of the vestibulo-ocular reflex--around the flocculus hypothesis. *Annu Rev Neurosci,* 5**,** 275-96.

ITO, M. 1984. *The Cerebellum and Neural Control,* New York, Raven Press.

ITO, M. 1989. Long-term depression. *Annu Rev Neurosci.,* 12**,** 85-102.

ITO, M. 2001. Cerebellar long-term depression: characterization, signal transduction, and functional roles. *Physiol Rev,* 81**,** 1143-95.

ITO, M. 2002. The molecular organization of cerebellar long-term depression. *Nat Rev Neurosci,* 3**,** 896-902.

ITO, M. 2006. Cerebellar circuitry as a neuronal machine. *Prog Neurobiol,* 78**,** 272-303.

ITO, M. 2009. Functional roles of neuropeptides in cerebellar circuits. *Neuroscience,* 162**,** 666-72.

ITO, M. & KANO, M. 1982. Long-lasting depression of parallel fiber-Purkinje cell transmission induced by conjunctive stimulation of parallel fibers and climbing fibers in the cerebellar cortex. *Neurosci Lett,* 33**,** 253-258.

ITO, M., SHIIDA, T., YAGI, N. & YAMAMOTO, M. 1974. Visual influence on rabbit horizontal vestibulo-ocular reflex presumably effected via the cerebellar flocculus. *Brain Res,* 65**,** 170-4.

ITO, M., JASTREBOFF, P. J. & MIYASHITA, Y. 1980. Retrograde influence of surgical and chemical flocculectomy upon dorsal cap neurons of the inferior olive. *Neurosci Lett,* 20**,** 45-48.

ITO, M., JASTREBOFF, P. J. & MIYASHITA, Y. 1981. Specific effects of unilateral lesions in the flocculus upon eye movements in albino rabbits. *Exp Brain Res,* 45**,** 233-42.

ITO, M., SAKURAI, M. & TONGROACH, P. 1982. Climbing fibre induced depression of both mossy fibre responsiveness and glutamate sensitivity of cerebellar Purkinje cells. *J Physiol,* 324**,** 113-134.

IZQUIERDO, I., MEDINA, J. H., IZQUIERDO, L. A., BARROS, D. M., DE SOUZA, M. M. & MELLO E SOUZA, T. 1998. Short- and Long-Term Memory Are Differentially Regulated by Monoaminergic Systems in the Rat Brain. *Neurobiol Learn Mem,* 69**,** 219-224.

JAARSMA, D., RUIGROK, T. J., CAFFE, R., COZZARI, C., LEVEY, A. I., MUGNAINI, E. & VOOGD, J. 1997. Cholinergic innervation and receptors in the cerebellum. *Prog Brain Res,* 114**,** 67-96.

JAKAB, R. L. & HAMORI, J. 1988. Quantitative morphology and synaptology of cerebellar glomeruli in the rat. *Anat Embryol (Berl),* 179**,** 81-8.

JI, J.-Z., WANG, X.-M. & LI, B.-M. 2003. Deficit in long-term contextual fear memory induced by blockade of β-adrenoceptors in hippocampal CA1 region. *Eur J Neurosci,* 17**,** 1947-1952.

JIRENHED, D. A., BENGTSSON, F. & HESSLOW, G. 2007. Acquisition, extinction, and reacquisition of a cerebellar cortical memory trace. *J Neurosci,* 27**,** 2493-502.

JIRENHED, D. A. & HESSLOW, G. 2015. Are Purkinje Cell Pauses Drivers of Classically Conditioned Blink Responses? *Cerebellum***,** 1-9.

JOHANSSON, F., JIRENHED, D.-A., RASMUSSEN, A., ZUCCA, R. & HESSLOW, G. 2014. Memory trace and timing mechanism localized to cerebellar Purkinje cells. *Proc Natl Acad Sci U S A,* 111**,** 14930-14934.

JOHANSSON, F., CARLSSON, H. A., RASMUSSEN, A., YEO, C. H. & HESSLOW, G. 2015. Activation of a Temporal Memory in Purkinje Cells by the $mGluR_7$ Receptor. *Cell Rep,* 13**,** 1741-6.

JOHANSSON, F., HESSLOW, G. & MEDINA, J. F. 2016. Mechanisms for motor timing in the cerebellar cortex. *Curr Op Beh Sci,* 8**,** 53-59.

JOHNSON, E. W., WOLFE, B. B. & MOLINOFF, P. B. 1989. Regulation of subtypes of beta-adrenergic receptors in rat brain following treatment with 6-hydroxydopamine. *J Neurosci,* 9**,** 2297-305.

JONES, E. G. 1975. Possible determinants of the degree of retrograde neuronal labeling with horseradish peroxidase. *Brain Res,* 85**,** 249-253.

JORNTELL, H. & EKEROT, C. F. 2002. Reciprocal bidirectional plasticity of parallel fiber receptive fields in cerebellar Purkinje cells and their afferent interneurons. *Neuron,* 34**,** 797-806.

JORNTELL, H. & EKEROT, C. F. 2003. Receptive field plasticity profoundly alters the cutaneous parallel fiber synaptic input to cerebellar interneurons in vivo. *J Neurosci,* 23**,** 9620-31.

JORNTELL, H. & EKEROT, C. F. 2006. Properties of somatosensory synaptic integration in cerebellar granule cells in vivo. *J Neurosci,* 26**,** 11786-97.

JORNTELL, H., BENGTSSON, F., SCHONEWILLE, M. & DE ZEEUW, C. I. 2010. Cerebellar molecular layer interneurons - computational properties and roles in learning. *Trends Neurosci,* 33**,** 524-32.

KALINICHENKO, S. G. & OKHOTIN, V. E. 2005. Unipolar brush cells--a new type of excitatory interneuron in the cerebellar cortex and cochlear nuclei of the brainstem. *Neurosci Behav Physiol,* 35**,** 21-36.

KANDEL, ERIC R., DUDAI, Y. & MAYFORD, MARK R. 2014. The Molecular and Systems Biology of Memory. *Cell,* 157**,** 163-186.

KANICHAY, R. T. & SILVER, R. A. 2008. Synaptic and cellular properties of the feedforward inhibitory circuit within the input layer of the cerebellar cortex. *J Neurosci,* 28**,** 8955-67.

KARACHOT, L., KADO, R. T. & ITO, M. 1994. Stimulus parameters for induction of long-term depression in in vitro rat Purkinje cells. *Neurosci Res,* 21**,** 161-168.

KAWATO, M. & GOMI, H. 1992. The cerebellum and VOR/OKR learning models. *Trends Neurosci,* 15**,** 445-453.

KELLETT, D. O., FUKUNAGA, I., CHEN-KUBOTA, E., DEAN, P. & YEO, C. H. 2010. Memory consolidation in the cerebellar cortex. *PLoS One,* 5**,** e11737.

KELLETT, D. O. & YEO, C. H. 2007. A noradrenergic mechanism of motor memory consolidation in the cerebellar cortex. *Proceedings of the British Pharmacological Society,* 5, abstract 150P. Found at *http://www.pA2online.org/abstracts/Vol5Issue2abst140P.pdf*

KERR, C. W. & BISHOP, G. A. 1991. Topographical organization in the origin of serotoninergic projections to different regions of the cat cerebellar cortex. *J Comp Neurol,* 304**,** 502-15.

KERR, C. W. & BISHOP, G. A. 1992. The physiological effects of serotonin are mediated by the 5-HT_{1A} receptor in the cat's cerebellar cortex. *Brain Res,* 591**,** 253-60.

KETY, S. S. 1970. The biogenic amines in the central nervous system: their possible roles in arousal, emotion and learning. *In:* SCHMIDT, F. O. (ed.) *The neurosciences: second study program.* New York: Rockefellar Press.

KIA, H. K., MIQUEL, M.-C., BRISORGUEIL, M.-J., DAVAL, G., RIAD, M., MESTIKAWY, S. E., HAMON, M. & VERGÉ, D. 1996. Immunocytochemical localization of serotonin1A receptors in the rat central nervous system. *J Comp Neurol,* 365**,** 289-305.

KIMOTO, Y., TOHYAMA, M., SATOH, K., SAKUMOTO, T., TAKAHASHI, Y. & SHIMIZU, N. 1981. Fine structure of rat cerebellar noradrenaline terminals as visualized by potassium permanganate 'in situ perfusion' fixation method. *Neuroscience,* 6**,** 47-58.

KING, M. V., MARSDEN, C. A. & FONE, K. C. F. 2008. A role for the 5-HT_{1A}, 5-HT_4 and 5-HT_6 receptors in learning and memory. *Trends Pharmacol Sci,* 29**,** 482-492.

KING, V. M., ARMSTRONG, D. M., APPS, R. & TROTT, J. R. 1998. Numerical aspects of pontine, lateral reticular, and inferior olivary projections to two paravermal cortical zones of the cat cerebellum. *J Comp Neurol,* 390**,** 537-51.

KITZMAN, P. H. & BISHOP, G. A. 1994. The origin of serotoninergic afferents to the cat's cerebellar nuclei. *J Comp Neurol,* 340**,** 541-50.

KOEKKOEK, S. K., HULSCHER, H. C., DORTLAND, B. R., HENSBROEK, R. A., ELGERSMA, Y., RUIGROK, T. J. & DE ZEEUW, C. I. 2003. Cerebellar LTD and learning-dependent timing of conditioned eyelid responses. *Science,* 301**,** 1736-9.

KONDO, S. & MARTY, A. 1998. Differential effects of noradrenaline on evoked, spontaneous and miniature IPSCs in rat cerebellar stellate cells. *J Physiol,* 509 (Pt 1)**,** 233-43.

KOZIOL, L. F., BUDDING, D., ANDREASEN, N., D'ARRIGO, S., BULGHERONI, S., IMAMIZU, H., ITO, M., MANTO, M., MARVEL, C., PARKER, K., PEZZULO, G.,

RAMNANI, N., RIVA, D., SCHMAHMANN, J., VANDERVERT, L. & YAMAZAKI, T. 2013. Consensus Paper: The Cerebellum's Role in Movement and Cognition. *Cerebellum,* 13**,** 151-177.

KRUPA, D. J. & THOMPSON, R. F. 1995. Inactivation of the superior cerebellar peduncle blocks expression but not acquisition of the rabbit's classically conditioned eye-blink response. *Proc Natl Acad Sci U S A.,* 92**,** 5097-5101.

KRUPA, D. J., THOMPSON, J. K. & THOMPSON, R. F. 1993. Localization of a memory trace in the mammalian brain. *Science,* 260**,** 989-91.

LAINE, J. & AXELRAD, H. 1998. Lugaro cells target basket and stellate cells in the cerebellar cortex. *Neuroreport,* 9**,** 2399-403.

LAINE, J. & AXELRAD, H. 2002. Extending the cerebellar Lugaro cell class. *Neuroscience,* 115**,** 363-74.

LANDIS, S. C., SHOEMAKER, W. J., SCHLUMPF, M. & BLOOM, F. E. 1975. Catecholamines in mutant mouse cerebellum: fluorescence microscopic and chemical studies. *Brain Res,* 93**,** 253-66.

LARSELL, O. 1952. The morphogenesis and adult pattern of the lobules and fissures of the cerebellum of the white rat. *J Comp Neurol,* 97**,** 281-356.

LARSELL, O. 1953. The cerebellum of the cat and the monkey. *J Comp Neurol,* 99**,** 135-99.

LAVOND, D. G., MCCORMICK, D. A., CLARK, G. A., HOLMES, D. T. & THOMPSON, R. F. 1981. Effects of ipsilateral rostral pontine reticular lesions on retention of classically conditioned nictitating membrane and eyelid responses. *Physiological Psychology,* 9**,** 5.

LAVOND, D. G., STEINMETZ, J. E., YOKAITIS, M. H. & THOMPSON, R. F. 1987. Reacquisition of classical conditioning after removal of cerebellar cortex. *Exp Brain Res,* 67**,** 569-93.

LE MAREC, N., ASE, A. R., BOTEZ-MARQUARD, T., MARCHAND, L., READER, T. A. & LALONDE, R. 2001. Behavioral and biochemical effects of L-tryptophan and buspirone in a model of cerebellar atrophy. *Pharmacol Biochem Behav,* 69**,** 333-42.

LEE, E. H., LIN, W. R., CHEN, H. Y., SHIU, W. H. & LIANG, K. C. 1992. Fluoxetine and 8-OH-DPAT in the lateral septum enhances and impairs retention of an inhibitory avoidance response in rats. *Physiol Behav,* 51**,** 681-8.

LEE, M., STRAHLENDORF, J. C. & STRAHLENDORF, H. K. 1985. Modulatory action of serotonin on glutamate-induced excitation of cerebellar Purkinje cells. *Brain Res,* 361**,** 107-13.

LEPORA, N. F., PORRILL, J., YEO, C. & DEAN, P. 2010. Sensory prediction or motor control? Application of Marr-Albus type models of cerebellar function to classical conditioning. *Frontiers in Computational Neuroscience,* 4.

LEUNER, B., MENDOLIA-LOFFREDO, S. & SHORS, T. J. 2004. Males and Females Respond Differently to Controllability and Antidepressant Treatment. *Biol Psychiatry,* 56**,** 964-970.

LIANG, K. C. 1999. Pre- or post-training injection of buspirone impaired retention in the inhibitory avoidance task: involvement of amygdala 5-HT_{1A} receptors. Eur J *Neurosci,* 11**,** 1491-1500.

LIANG, K. C., JULER, R. G. & MCGAUGH, J. L. 1986. Modulating effects of posttraining epinephrine on memory: involvement of the amygdala noradrenergic system. *Brain Res,* 368**,** 125-33.

LIEBEN, C. K. J., OORSOUW, K. V., DEUTZ, N. E. P. & BLOKLAND, A. 2004. Acute tryptophan depletion induced by a gelatin-based mixture impairs object memory but not affective behavior and spatial learning in the rat. *Beh Brain Res,* 151**,** 53-64.

LINCOLN, J. S., MCCORMICK, D. A. & THOMPSON, R. F. 1982. Ipsilateral cerebellar lesions prevent learning of the classically conditioned nictitating membrane/eyelid response. *Brain Res,* 242**,** 190-3.

LIPPIELLO, P., HOXHA, E., VOLPICELLI, F., LO DUCA, G., TEMPIA, F. & MINIACI, M. C. 2015. Noradrenergic modulation of the parallel fiber-Purkinje cell synapse in mouse cerebellum. *Neuropharmacology,* 89**,** 33-42.

LIPPIELLO, P., HOXHA, E., SPERANZA, L., VOLPICELLI, F., FERRARO, A., LEOPOLDO, M., LACIVITA, E., PERRONE-CAPANO, C., TEMPIA, F. & MINIACI, M. C. 2016. The 5-HT_7 receptor triggers cerebellar long-term synaptic depression via PKC-MAPK. *Neuropharmacology,* 101**,** 426-38.

LLANO, I. & GERSCHENFELD, H. M. 1993. Beta-adrenergic enhancement of inhibitory synaptic activity in rat cerebellar stellate and Purkinje cells. *J Physiol,* 468**,** 201-24.

LLINAS, R., WALTON, K. D. & LANG, E. J. 2004. Cerebellum. *In:* SHEPHERD, G. M. (ed.) *The Synaptic Organisation of the Brain.* 5 ed. New York: Oxford University Press.

LONGLEY, M. & YEO, C. H. 2014. Distribution of neural plasticity in cerebellum-dependent motor learning. *Prog Brain Res.,* 210**,** 79-101.

LOUGHLIN, S. E., FOOTE, S. L. & BLOOM, F. E. 1986a. Efferent projections of nucleus locus coeruleus: Topographic organization of cells of origin demonstrated by three-dimensional reconstruction. *Neuroscience,* 18**,** 291-306.

LOUGHLIN, S. E., FOOTE, S. L. & GRZANNA, R. 1986b. Efferent projections of nucleus locus coeruleus: Morphologic subpopulations have different efferent targets. *Neuroscience,* 18**,** 307-319.

LU, H., ESQUIVEL, A. V. & BOWER, J. M. 2009. 3D electron microscopic reconstruction of segments of rat cerebellar purkinje cell dendrites receiving ascending and parallel fiber granule cell synaptic inputs. *J Comp Neurol,* 514**,** 583-594.

LÜTTGEN, M., ELVANDER, E., MADJID, N. & ÖGREN, S. O. 2005. Analysis of the role of 5-HT_{1A} receptors in spatial and aversive learning in the rat. *Neuropharmacology,* 48**,** 830-852.

MAESHIMA, T., SHUTOH, F., HAMADA, S., SENZAKI, K., HAMAGUCHI-HAMADA, K., ITO, R. & OKADO, N. 1998. Serotonin2A receptor-like immunoreactivity in rat cerebellar Purkinje cells. *Neurosci Lett,* 252**,** 72-4.

MARR, D. 1969. A theory of cerebellar cortex. *J Physiol,* 202**,** 437-470.

MARZO, A., BAI, J. & OTANI, S. 2009. Neuroplasticity regulation by noradrenaline in mammalian brain. *Curr Neuropharmacol,* 7**,** 286-95.

MATSUI, K. & JAHR, C. E. 2004. Differential control of synaptic and ectopic vesicular release of glutamate. *J Neurosci,* 24**,** 8932-9.

MATSUI, K., JAHR, C. E. & RUBIO, M. E. 2005. High-concentration rapid transients of glutamate mediate neural-glial communication via ectopic release. *J Neurosci,* 25**,** 7538-47.

MATYASH, V., FILIPPOV, V., MOHRHAGEN, K. & KETTENMANN, H. 2001. Nitric oxide signals parallel fiber activity to Bergmann glial cells in the mouse cerebellar slice. *Mol Cell Neurosci,* 18**,** 664-70.

MAUK, M. D. & THOMPSON, R. F. 1987. Retention of classically conditioned eyelid responses following acute decerebration. *Brain Res.,* 403**,** 89-95.

MAURA, G., RICCHETTI, A. & RAITERI, M. 1986. Serotonin inhibits the depolarization-evoked release of endogenous glutamate from rat cerebellar nerve endings. *Neurosci Lett,* 67**,** 218-22.

MAURA, G., ROCCATAGLIATA, E., ULIVI, M. & RAITERI, M. 1988. Serotonin-glutamate interaction in rat cerebellum: involvement of 5-HT_1 and 5-HT_2 receptors. *Eur J Pharmacol,* 145**,** 31-8.

MCCORMICK, D. A. & THOMPSON, R. F. 1984. Cerebellum: essential involvement in the classically conditioned eyelid response. *Science,* 223**,** 296-9.

MCCORMICK, D. A., LAVOND, D. G., CLARK, G. A., KETTNER, R. E., RISING, C. E. & THOMPSON, R. F. 1981. The engram found? Role of the cerebellum in classical conditioning of nictitating membrane and eyelid responses. *Bulletin of the Psychonomic Society,* 18**,** 3.

MCCORMICK, D. A., CLARK, G. A., LAVOND, D. G. & THOMPSON, R. F. 1982a. Initial localization of the memory trace for a basic form of learning. *Proc Natl Acad Sci U S A,* 79, 2731-5.

MCCORMICK, D. A., GUYER, P. E. & THOMPSON, R. F. 1982b. Superior cerebellar peduncle lesions selectively abolish the ipsilateral classically conditioned nictitating membrane/eyelid response of the rabbit. *Brain Res,* 244**,** 347-50.

MCGAUGH, J. L. 2004. The amygdala modulates the consolidation of memories of emotionally arousing experiences. *Annu Rev Neurosci,* 27**,** 1-28.

MELAMED, E., LAHAV, M. & ATLAS, D. 1976. Direct localisation of β-adrenoceptor sites in rat cerebellum by a new fluorescent analogue of propranolol. *Nature,* 261**,** 3.

MELCHITZKY, D. S. & LEWIS, D. A. 2000. Tyrosine Hydroxylase- and Dopamine Transporter-Immunoreactive Axons in the Primate Cerebellum. *Neuropsychopharmacology,* 22**,** 466-472.

MENDLIN, A., MARTIN, F. J., RUETER, L. E. & JACOBS, B. L. 1996. Neuronal release of serotonin in the cerebellum of behaving rats: an in vivo microdialysis study. *J Neurochem,* 67**,** 617-22.

MENESES, A. & HONG, E. 1995. Effect of fluoxetine on learning and memory involves multiple 5-HT systems. *Pharmacol Biochem Behav,* 52**,** 341-6.

MENESES, A. & PEREZ-GARCIA, G. 2007. 5-HT(1A) receptors and memory. *Neurosci Biobehav Rev,* 31**,** 705-27.

MENESES, A. & LIY-SALMERON, G. 2012. Serotonin and emotion, learning and memory. *Rev Neurosci,* 23**,** 543-53.

MIHAILOFF, G. A. 1993. Cerebellar nuclear projections from the basilar pontine nuclei and nucleus reticularis tegmenti pontis as demonstrated with PHA-L tracing in the rat. *J Comp Neurol,* 330**,** 130-146.

MIQUEL, M. C., KIA, H. K., BONI, C., DOUCET, E., DAVAL, G., MATTHIESSEN, L., HAMON, M. & VERGE, D. 1994. Postnatal development and localization of 5-HT_{1A} receptor mRNA in rat forebrain and cerebellum. *Brain Res Brain Res Rev,* 80**,** 149-57.

MITOMA, H. & KONISHI, S. 1999. Monoaminergic long-term facilitation of GABA-mediated inhibitory transmission at cerebellar synapses. *Neuroscience,* 88**,** 871-83.

MOISES, H. C., WOODWARD, D. J., HOFFER, B. J. & FREEDMAN, R. 1979. Interactions of norepinephrine with Purkinje cell responses to putative amino acid neurotransmitters applied by microiontophoresis. *Exp Neurol,* 64**,** 493-515.

MONTAROLO, P. G., PALESTINI, M. & STRATA, P. 1982. The inhibitory effect of the olivocerebellar input on the cerebellar Purkinje cells in the rat. *J Physiol* 332**,** 187-202.

MOORE, J. W., DESMOND, J. E. & BERTHIER, N. E. 1989. Adaptively timed conditioned responses and the cerebellum: a neural network approach. *Biol Cybern.,* 62**,** 17-28.

MOSTOFI, A., HOLTZMAN, T., GROUT, A. S., YEO, C. H. & EDGLEY, S. A. 2010. Electrophysiological localization of eyeblink-related microzones in rabbit cerebellar cortex. *J Neurosci,* 30**,** 8920-34.

MUGNAINI, E. & DAHL, A. L. 1975. Mode of distribution of aminergic fibers in the cerebellar cortex of the chicken. *J Comp Neurol,* 162**,** 417-32.

MURANO, M., SAITOW, F. & SUZUKI, H. 2011. Modulatory effects of serotonin on glutamatergic synaptic transmission and long-term depression in the deep cerebellar nuclei. *Neuroscience,* 172**,** 118-28.

MURRIN, L. C., SANDERS, J. D. & BYLUND, D. B. 2007. Comparison of the maturation of the adrenergic and serotonergic neurotransmitter systems in the brain: implications for differential drug effects on juveniles and adults. *Biochem Pharmacol,* 73**,** 1225-36.

NAGAI, T., SATOH, K., IMAMOTO, K. & MAEDA, T. 1981. Divergent projections of catecholamine neurons of the locus coeruleus as revealed by fluorescent retrograde double labeling technique. *Neurosci Lett,* 23**,** 117-23.

NAGAO, S. 1983. Effects of vestibulocerebellar lesions upon dynamic characteristics and adaptation of vestibulo-ocular and optokinetic responses in pigmented rabbits. *Exp Brain Res,* 53**,** 36-46.

NAPPER, R. M. & HARVEY, R. J. 1988. Number of parallel fiber synapses on an individual Purkinje cell in the cerebellum of the rat. *J Comp Neurol,* 274**,** 168-77.

NELSON, B. & MUGNAINI, E. 1989. Origins of GABA-ergic inputs to the inferior olive. *In:* STRATA, P. (ed.) *The olivocerebellar systems in motor control.* Berlin: Springer-Verlag.

NICHOLAS, A. P., PIERIBONE, V. A. & HÖKFELT, T. 1993. Cellular localization of messenger RNA for beta-1 and beta-2 adrenergic receptors in rat brain: An in situ hybridization study. *Neuroscience,* 56**,** 1023-1039.

NICHOLS, R. A. 2011. Serotonin, presynaptic 5-HT(3) receptors and synaptic plasticity in the developing cerebellum. *J Physiol,* 589**,** 5019-20.

NIELSEN, K., BRASK, D., KNUDSEN, G. M. & AZNAR, S. 2006. Immunodetection of the serotonin transporter protein is a more valid marker for serotonergic fibers than serotonin. *Synapse,* 59**,** 270-6.

NILAWEERA, W. U., IRWIN, K. B., D., Z. G., AKSENOV, D. P. & BRACHA, V. 2002. Are feedback loops involved in the formulation of cerebellar neuronal correlates of conditioned eyeblinks? *Soc. Neurosci. Abstr. ,* 79**,** 6.

NIMMERJAHN, A., MUKAMEL, E. A. & SCHNITZER, M. J. 2009. Motor Behavior Activates Bergmann Glial Networks. *Neuron,* 62**,** 400.

NISHIDA, H. & OKABE, S. 2007. Direct Astrocytic Contacts Regulate Local Maturation of Dendritic Spines. *J Neurosci,* 27**,** 331-340.

OAKLEY, D. A. & RUSSELL, I. S. 1972. Neocortical lesions and Pavlovian conditioning. *Physiol Behav.,* 8**,** 915-926.

OAKLEY, D. A. & RUSSELL, I. S. 1975. Role of cortex in Pavlovian discrimination learning. *Physiol Behav.,* 15**,** 315-321.

OAKLEY, D. A. & RUSSELL, I. S. 1977. Subcortical storage of Pavlovian conditioning in the rabbit. *Physiol Behav.,* 18**,** 931-937.

OGREN, S. O. 1985. Evidence for a role of brain serotonergic neurotransmission in avoidance learning. *Acta Physiol Scand Suppl,* 544**,** 1-71.

OGREN, S. O., ERIKSSON, T. M., ELVANDER-TOTTIE, E., D'ADDARIO, C., EKSTROM, J. C., SVENNINGSSON, P., MEISTER, B., KEHR, J. & STIEDL, O. 2008. The role of 5-HT(1A) receptors in learning and memory. *Behav Brain Res,* 195**,** 54-77.

OHMAE, S. & MEDINA, J. F. 2015. Climbing fibers encode a temporal-difference prediction error during cerebellar learning in mice. *Nat Neurosci,* 18**,** 1798-803.

OKAMOTO, T., ENDO, S., SHIRAO, T. & NAGAO, S. 2011a. Role of cerebellar cortical protein synthesis in transfer of memory trace of cerebellum-dependent motor learning. *J Neurosci,* 31**,** 8958-66.

OKAMOTO, T., SHIRAO, T., SHUTOH, F., SUZUKI, T. & NAGAO, S. 2011b. Post-training cerebellar cortical activity plays an important role for consolidation of memory of cerebellum-dependent motor learning. *Neurosci Lett,* 504**,** 53-6.

OLIET, S. H. R., PIET, R. & POULAIN, D. A. 2001. Control of Glutamate Clearance and Synaptic Efficacy by Glial Coverage of Neurons. *Science,* 292**,** 923-926.

OLIVER, K. R., KINSEY, A. M., WAINWRIGHT, A. & SIRINATHSINGHJI, D. J. S. 2000. Localization of 5-HT_{5A} receptor-like immunoreactivity in the rat brain. *Brain Res,* 867**,** 131-142.

OLSON, L. & FUXE, K. 1971. On the projections from the locus coeruleus noradrealine neurons: the cerebellar innervation. *Brain Res,* 28**,** 165-71.

OOSTLAND, M., SELLMEIJER, J. & VAN HOOFT, J. A. 2011. Transient expression of functional serotonin 5-HT_3 receptors by glutamatergic granule cells in the early postnatal mouse cerebellum. *J Physiol,* 589**,** 4837-46.

OOSTLAND, M., BUIJINK, M. R. & VAN HOOFT, J. A. 2013. Serotonergic control of Purkinje cell maturation and climbing fibre elimination by 5-HT_3 receptors in the juvenile mouse cerebellum. *J Physiol,* 591**,** 1793-807.

OOSTLAND, M., BUIJINK, M. R., TEUNISSE, G. M., VON OERTHEL, L., SMIDT, M. P. & VAN HOOFT, J. A. 2014. Distinct temporal expression of 5-HT(1A) and 5-HT(2A) receptors on cerebellar granule cells in mice. *Cerebellum,* 13**,** 491-500.

OSCARSSON, O. 1979. Functional units of the cerebellum - sagittal zones and microzones. *Trends Neurosci,* 2**,** 3.

PALACIOS, J. & KUHAR, M. J. 1982. Beta adrenergic receptor localization in rat brain by light microscopic autoradiography. *Neurochem Int,* 4**,** 473-90.

PALACIOS, J. M., HOYER, D. & CORTÉS, R. 1987. α_1-adrenoceptors in the mammalian brain: similar pharmacology but different distribution in rodents and primates. *Brain Res,* 419**,** 65-75.

PALAY, S. L. & CHAN-PALAY, V. 1974. *Cerebellar Cortex: Cytology and Organization,* Berlin, Springer-Verlag.

PANATIER, A., THEODOSIS, D. T., MOTHET, J.-P., TOUQUET, B., POLLEGIONI, L., POULAIN, D. A. & OLIET, S. H. R. 2006. Glia-Derived d-Serine Controls NMDA Receptor Activity and Synaptic Memory. *Cell,* 125**,** 775-784.

PANULA, P., PIRVOLA, U., AUVINEN, S. & AIRAKSINEN, M. S. 1989. Histamine-immunoreactive nerve fibers in the rat brain. *Neuroscience,* 28**,** 585-610.

PANULA, P., TAKAGI, H., INAGAKI, N., YAMATODANI, A., TOHYAMA, M., WADA, H. & KOTILAINEN, E. 1993. Histamine-containing nerve fibers innervate human cerebellum. *Neurosci Lett,* 160**,** 53-56.

PAPADOPOULOS, G. C. & PARNAVELAS, J. G. 1990. Distribution and synaptic organization of serotoninergic and noradrenergic axons in the lateral geniculate nucleus of the rat. *J Comp Neurol,* 294**,** 345-55.

PAPADOPOULOS, G. C., PARNAVELAS, J. G. & BUIJS, R. M. 1989. Light and electron microscopic immunocytochemical analysis of the noradrenaline innervation of the rat visual cortex. *J Neurocytol,* 18**,** 1-10.

PAPAY, R., GAIVIN, R., MCCUNE, D. F., RORABAUGH, B. R., MACKLIN, W. B., MCGRATH, J. C. & PEREZ, D. M. 2004. Mouse alpha1B-adrenergic receptor is expressed in neurons and NG2 oligodendrocytes. *J Comp Neurol,* 478**,** 1-10.

PAPAY, R., GAIVIN, R., JHA, A., MCCUNE, D. F., MCGRATH, J. C., RODRIGO, M. C., SIMPSON, P. C., DOZE, V. A. & PEREZ, D. M. 2006. Localization of the mouse alpha1A-adrenergic receptor (AR) in the brain: alpha1AAR is expressed in neurons, GABAergic interneurons, and NG2 oligodendrocyte progenitors. *J Comp Neurol,* 497**,** 209-22.

PAREDES, D. A., CARTFORD, M. C., CATLOW, B. J., SAMEC, A., AVILAS, M., GEORGE, A., SCHLUNCK, A., SMALL, B. & BICKFORD, P. C. 2009. Neurotransmitter release during delay eyeblink classical conditioning: role of norepinephrine in consolidation and effect of age. *Neurobiol Learn Mem,* 92**,** 267-82.

PASCHALIS, A., CHURCHILL, L., MARINA, N., KASYMOV, V., GOURINE, A. & ACKLAND, G. 2009. beta1-Adrenoceptor distribution in the rat brain: an immunohistochemical study. *Neurosci Lett,* 458**,** 84-8.

PASQUALETTI, M., ORI, M., CASTAGNA, M., MARAZZITI, D., CASSANO, G. B. & NARDI, I. 1999. Distribution and cellular localization of the serotonin type 2C receptor messenger RNA in human brain. *Neuroscience,* 92**,** 601-11.

PASQUIER, D. A., GOLD, M. A. & JACOBOWITZ, D. M. 1980. Noradrenergic perikarya (A5-A7, subcoeruleus) projections to the rat cerebellum. *Brain Res,* 196**,** 270-5.

PAUKERT, M., AGARWAL, A., CHA, J., DOZE, VAN A., KANG, JIN U. & BERGLES, DWIGHT E. 2014. Norepinephrine Controls Astroglial Responsiveness to Local Circuit Activity. *Neuron,* 82**,** 1263-1270.

PERRETT, S. P. & MAUK, M. D. 1995. Extinction of conditioned eyelid responses requires the anterior lobe of cerebellar cortex. *J Neurosci,* 15**,** 2074-80.

PERRETT, S. P., RUIZ, B. P. & MAUK, M. D. 1993. Cerebellar cortex lesions disrupt learning-dependent timing of conditioned eyelid responses. *J Neurosci,* 13**,** 1708-18.

PICHITPORNCHAI, C., RAWSON, J. A. & REES, S. 1994. Morphology of parallel fibres in the cerebellar cortex of the rat: an experimental light and electron microscopic study with biocytin. *J Comp Neurol,* 342**,** 206-20.

PICKEL, V. M., SEGAL, M. & BLOOM, F. E. 1974. A radioautographic study of the efferent pathways of the nucleus locus coeruleus. *J Comp Neurol,* 155**,** 15-42.

PIERIBONE, V. A., NICHOLAS, A. P., DAGERLIND, A. & HÖKFELT, T. 1994. Distribution of alpha 1 adrenoceptors in rat brain revealed by in situ hybridization experiments utilizing subtype-specific probes. *J Neurosci,* 14**,** 4252-68.

PIET, R., POULAIN, D. A. & OLIET, S. H. R. 2004. Contribution of astrocytes to synaptic transmission in the rat supraoptic nucleus. *Neurochem Int,* 45**,** 251-257.

PIET, R. & JAHR, C. E. 2007. Glutamatergic and Purinergic Receptor-Mediated Calcium Transients in Bergmann Glial Cells. *J Neurosci,* 27**,** 4027-4035.

PIJPERS, A., APPS, R., PARDOE, J., VOOGD, J. & RUIGROK, T. J. 2006. Precise spatial relationships between mossy fibers and climbing fibers in rat cerebellar cortical zones. *J Neurosci,* 26**,** 12067-80.

PITSIKAS, N., RIGAMONTI, A. E., CELLA, S. G. & MULLER, E. E. 2003. The 5-HT_{1A} receptor antagonist WAY 100635 improves rats performance in different models of amnesia evaluated by the object recognition task. *Brain Res,* 983**,** 215-22.

POLLARD, H., MOREAU, J., ARRANG, J. M. & SCHWARTZ, J. C. 1993. A detailed autoradiographic mapping of histamine H3 receptors in rat brain areas. *Neuroscience,* 52**,** 169-189.

POMPEIANO, O. 1998. Noradrenergic influences on the cerebellar cortex: effects on vestibular reflexes under basic and adaptive conditions. *Otolaryngol Head Neck Surg,* 119**,** 93-105.

PORRILL, J., DEAN, P. & ANDERSON, S. R. 2013. Adaptive filters and internal models: Multilevel description of cerebellar function. *Neural Networks,* 47**,** 134-149.

PRADO-ALCALA, R. A., RUILOBA, M. I., RUBIO, L., SOLANA-FIGUEROA, R., MEDINA, C., SALADO-CASTILLO, R. & QUIRARTE, G. L. 2003. Regional infusions of serotonin into the striatum and memory consolidation. *Synapse,* 47**,** 169-75.

PRESTORI, F., BONARDI, C., MAPELLI, L., LOMBARDO, P., GOSELINK, R., DE STEFANO, M. E., GANDOLFI, D., MAPELLI, J., BERTRAND, D., SCHONEWILLE, M., DE ZEEUW, C. & D'ANGELO, E. 2013. Gating of Long-Term Potentiation by Nicotinic Acetylcholine Receptors at the Cerebellum Input Stage. *PLoS ONE,* 8**,** e64828.

QIAN, Y., MELIKIAN, H. E., RYE, D. B., LEVEY, A. I. & BLAKELY, R. D. 1995. Identification and characterization of antidepressant-sensitive serotonin transporter proteins using site-specific antibodies. *J Neurosci,* 15**,** 1261-74.

RAINBOW, T. C., PARSONS, B. & WOLFE, B. B. 1984. Quantitative autoradiography of beta 1- and beta 2-adrenergic receptors in rat brain. *Proc Natl Acad Sci U S A,* 81**,** 1585-9.

RAMNANI, N. & YEO, C. H. 1996. Reversible inactivations of the cerebellum prevent the extinction of conditioned nictitating membrane responses in rabbits. *J Physiol,* 495 (Pt 1)**,** 159-68.

RASUL, A., JOHANSSON, B., LONNE-RAHM, S. B., NORDLIND, K., THEODORSSON, E. & EL-NOUR, H. 2013. Chronic mild stress modulates 5-HT_{1A} and 5-HT_{2A} receptor expression in the cerebellar cortex of NC/Nga atopic-like mice. *Arch Dermatol Res,* 305**,** 407-13.

RAWSON, J. A. & TILOKSKULCHAI, K. 1981. Suppression of simple spike discharges of cerebellar Purkinje cells by impulses in climbing fibre afferents. *Neurosci Lett,* 25**,** 125-130.

REICHENBACH, A., SIEGAL, A., RICKMANN, M., WOLFF, J. R. & NOONE, D. 1995. Distribution of Bergmann glia somata and processes: implications for function. *J Hirnforsch,* 36**,** 509-517.

RINALDO, L. & HANSEL, C. 2013. Muscarinic acetylcholine receptor activation blocks long-term potentiation at cerebellar parallel fiber–Purkinje cell synapses via cannabinoid signaling. *Proc Natl Acad Sci U S A,* 110**,** 11181-11186.

ROBERTS, A. J. & HEDLUND, P. B. 2012. The 5-HT(7) receptor in learning and memory. *Hippocampus,* 22**,** 762-71.

ROBERTSON, S. D., PLUMMER, N. W., DE MARCHENA, J. & JENSEN, P. 2013. Developmental origins of central norepinephrine neuron diversity. *Nat Neurosci,* 16**,** 1016-23.

ROBINSON, D. A. 1976. Adaptive gain control of vestibuloocular reflex by the cerebellum. *J Neurophysiol,* 39**,** 954-69.

ROGERS, D. C. & HAGAN, J. J. 2001. 5-HT_6 receptor antagonists enhance retention of a water maze task in the rat. *Psychopharmacology (Berl),* 158**,** 114-9.

ROMANO, A. G. & HARVEY, J. A. 1994. MDMA enhances associative and nonassociative learning in the rabbit. *Pharmacol Biochem Behav.,* 47**,** 289-293.

ROMANO, A. G., HOOD, H. & HARVEY, J. A. 2000. Dissociable effects of the 5-HT_2 antagonist mianserin on associative learning and performance in the rabbit. *Pharmacol Biochem Behav,* 67**,** 103-110.

ROSENFIELD, M. E. & MOORE, J. W. 1983. Red nucleus lesions disrupt the classically conditioned nictitating membrane response in rabbits. *Behav Brain Res,* 10**,** 393-98.

ROSENFIELD, M. E. & MOORE, J. W. 1985. Red nucleus lesions impair acquisition of the classically conditioned nictitating membrane response but not eye-to-eye savings or unconditioned response amplitude. *Behav Brain Res.,* 17**,** 77-81.

ROSENFIELD, M. E., DOVYDAITIS, A. & MOORE, J. W. 1985. Brachium conjuntivum and rubrobulbar tract: brain stem projections of red nucleus essential for the conditioned nictitating membrane response. *Physiol.Behav*, 34, 751-759

ROSIN, D. L., TALLEY, E. M., LEE, A., STORNETTA, R. L., GAYLINN, B. D., GUYENET, P. G. & LYNCH, K. R. 1996. Distribution of alpha 2C-adrenergic receptor-like immunoreactivity in the rat central nervous system. *J Comp Neurol,* 372**,** 135-65.

RUIGROK, T. J. 1997. Cerebellar nuclei: the olivary connection. *Prog Brain Res,,* 114**,** 167-192.

SAAB, A. S., NEUMEYER, A., JAHN, H. M., CUPIDO, A., ŠIMEK, A. A. M., BOELE, H.-J., SCHELLER, A., LE MEUR, K., GÖTZ, M., MONYER, H., SPRENGEL, R., RUBIO, M. E., DEITMER, J. W., DE ZEEUW, C. I. & KIRCHHOFF, F. 2012.

Bergmann Glial AMPA Receptors Are Required for Fine Motor Coordination. *Science,* 337**,** 749-753.

SAITOW, F. & KONISHI, S. 2000. Excitability increase induced by beta-adrenergic receptor-mediated activation of hyperpolarization-activated cation channels in rat cerebellar basket cells. *J Neurophysiol,* 84**,** 2026-34.

SAITOW, F., SATAKE, S., YAMADA, J. & KONISHI, S. 2000. beta-adrenergic receptor-mediated presynaptic facilitation of inhibitory GABAergic transmission at cerebellar interneuron-Purkinje cell synapses. *J Neurophysiol,* 84**,** 2016-25.

SAITOW, F., SUZUKI, H. & KONISHI, S. 2005. beta-Adrenoceptor-mediated long-term up-regulation of the release machinery at rat cerebellar GABAergic synapses. *J Physiol,* 565**,** 487-502.

SAITOW, F., MURANO, M. & SUZUKI, H. 2009. Modulatory effects of serotonin on GABAergic synaptic transmission and membrane properties in the deep cerebellar nuclei. *J Neurophysiol,* 101**,** 1361-74.

SAKURAI, M. 1987. Synaptic modification of parallel fibre-Purkinje cell transmission in in vitro guinea-pig cerebellar slices. *J Physiol,* 394**,** 463-480.

SALGADO, H., TREVIÑO, M. & ATZORI, M. Layer- and area-specific actions of norepinephrine on cortical synaptic transmission. *Brain Res*.

SALIN, P. A., MALENKA, R. C. & NICOLL, R. A. 1996. Cyclic AMP Mediates a Presynaptic Form of LTP at Cerebellar Parallel Fiber Synapses. *Neuron,* 16**,** 797-803.

SARA, S. J. 2015. Locus Coeruleus in time with the making of memories. *Curr Op Neurobiol,* 35**,** 87-94.

SARA, S. J. & SEGAL, M. 1991. Plasticity of sensory responses of locus coeruleus neurons in the behaving rat: implications for cognition. *Prog Brain Res,* 88, 571-585.

SARA, S. J. & BOURET, S. 2012. Orienting and reorienting: the locus coeruleus mediates cognition through arousal. *Neuron,* 76**,** 130-41.

SARA, S. J., VANKOV, A. & HERVE, A. 1994. Locus coeruleus-evoked responses in behaving rats: a clue to the role of noradrenaline in memory. *Brain Res Bull,* 35**,** 457-65.

SASAKI, T., BEPPU, K., TANAKA, K. F., FUKAZAWA, Y., SHIGEMOTO, R. & MATSUI, K. 2012. Application of an optogenetic byway for perturbing neuronal activity via glial photostimulation. *Proc Natl Acad Sci U S A,* 109**,** 20720-20725.

SCHAMBRA, U. B., MACKENSEN, G. B., STAFFORD-SMITH, M., HAINES, D. E. & SCHWINN, D. A. 2005. Neuron specific alpha-adrenergic receptor expression in human cerebellum: implications for emerging cerebellar roles in neurologic disease. *Neuroscience,* 135**,** 507-23.

SCHEININ, M., LOMASNEY, J. W., HAYDEN-HIXSON, D. M., SCHAMBRA, U. B., CARON, M. G., LEFKOWITZ, R. J. & FREMEAU, R. T., JR. 1994. Distribution of alpha 2-adrenergic receptor subtype gene expression in rat brain. *Brain Res Mol Brain Res,* 21**,** 133-49.

SCHINDELIN, J., ARGANDA-CARRERAS, I., FRISE, E., KAYNIG, V., LONGAIR, M., PIETZSCH, T., PREIBISCH, S., RUEDEN, C., SAALFELD, S., SCHMID, B., TINEVEZ, J.-Y., WHITE, D. J., HARTENSTEIN, V., ELICEIRI, K., TOMANCAK, P. & CARDONA, A. 2012. Fiji: an open-source platform for biological-image analysis. *Nat Meth,* 9**,** 676-682.

SCHMAHMANN, J. D. & SHERMAN, J. C. 1998. The cerebellar cognitive affective syndrome. *Brain,* 121 (Pt 4)**,** 561-79.

SCHNEIDER, A. M., WILKINS, E., FIRESTONE, A., EVERBACH, E. C., NAYLOR, J. C. & SIMSON, P. E. 2003. Enhanced Retention in the Passive-Avoidance Task By 5-HT(1A) Receptor Blockade Is Not Associated With Increased Activity of the Central Nucleus of the Amygdala. *Learn Mem,* 10**,** 394-400.

SCHNEIDERMAN, N., FUENTES, I. & GORMEZANO, I. 1962. Acquisition and extinction of the classically conditioned eyelid response in the albino rabbit. *Science,* 136**,** 650-2.

SCHONEWILLE, M., BELMEGUENAI, A., KOEKKOEK, S. K., HOUTMAN, S. H., BOELE, H. J., VAN BEUGEN, B. J., GAO, Z., BADURA, A., OHTSUKI, G., AMERIKA, W. E., HOSY, E., HOEBEEK, F. E., ELGERSMA, Y., HANSEL, C. & DE ZEEUW, C. I. 2010. Purkinje cell-specific knockout of the protein phosphatase PP2B impairs potentiation and cerebellar motor learning. *Neuron,* 67**,** 618-28.

SCHONEWILLE, M., GAO, Z., BOELE, H. J., VELOZ, M. F., AMERIKA, W. E., SIMEK, A. A., DE JEU, M. T., STEINBERG, J. P., TAKAMIYA, K., HOEBEEK, F. E., LINDEN, D. J., HUGANIR, R. L. & DE ZEEUW, C. I. 2011. Reevaluating the role of LTD in cerebellar motor learning. *Neuron,* 70**,** 43-50.

SCHROETER, S., APPARSUNDARAM, S., WILEY, R. G., MINER, L. H., SESACK, S. R. & BLAKELY, R. D. 2000. Immunolocalization of the cocaine- and antidepressant-sensitive l-norepinephrine transporter. *J Comp Neurol,* 420**,** 211-32.

SCHWEIGHOFER, N., DOYA, K. & KURODA, S. 2004. Cerebellar aminergic neuromodulation: towards a functional understanding. *Brain Res Rev,* 44**,** 103-16.

SEGAL, M., PICKEL, V. & BLOOM, F. 1973. The projections of the nucleus locus coeruleus: An autoradiographic study. *Life Sciences,* 13**,** 817-821.

SHINODA, Y., FUTAMI, T. & KANO, M. 1985a. Synaptic organization of the cerebello-thalamo-cerebral pathway in the cat. II. Input-output organization of single thalamocortical neurons in the ventrolateral thalamus. *Neurosci Res,* 2**,** 157-180.

SHINODA, Y., KANO, M. & FUTAMI, T. 1985b. Synaptic organization of the cerebello-thalamo-cerebral pathway in the cat. I. Projection of individual cerebellar nuclei to single pyramidal tract neurons in areas 4 and 6. *Neurosci Res,* 2**,** 133-156.

SHINODA, Y., SUGIUCHI, Y., FUTAMI, T. & IZAWA, R. 1992. Axon collaterals of mossy fibers from the pontine nucleus in the cerebellar dentate nucleus. *J Neurophysiol,* 67**,** 547-560.

SHINODA, Y., IZAWA, Y., SUGIUCHI, Y. & FUTAMI, T. 1997. Functional significance of excitatory projections from the precerebellar nuclei to interpositus and dentate nucleus neurons for mediating motor, premotor and parietal cortical inputs. *Prog Brain Res,* 114**,** 193-207.

SHUTOH, F., OHKI, M., KITAZAWA, H., ITOHARA, S. & NAGAO, S. 2006. Memory trace of motor learning shifts transsynaptically from cerebellar cortex to nuclei for consolidation. *Neuroscience,* 139**,** 767-77.

SIEGEL, S. & FREEDMAN, D. X. 1988. Effects of LSD-25 on classical trace conditioning. *Pharmacol Biochem Behav.,* 30**,** 427-431.

SILLITOE, R. V., CHUNG, S.-H., FRITSCHY, J.M., HOY, M. & HAWKES, R. 2008. Golgi Cell Dendrites Are Restricted by Purkinje Cell Stripe Boundaries in the Adult Mouse Cerebellar Cortex. *J Neurosci,* 28**,** 2820-2826.

SIMPSON, K. L. & LIN, R. C. S. 2007. Neuroanatomical and chemical organization of the locus coeruleus. *In:* ORDWAY, G. A., SCHWARTZ, M. A. & FRAZER, A. (eds.) *Brain Norepinephrine: Neurobiology and Therapeutics.* Cambridge, United Kingdom: Cambridge University Press.

SIMS, R. E. & HARTELL, N. A. 2005. Differences in transmission properties and susceptibility to long-term depression reveal functional specialization of ascending axon and parallel fiber synapses to Purkinje cells. *J Neurosci,* 25**,** 3246-57.

SIMS, R. E. & HARTELL, N. A. 2006. Differential susceptibility to synaptic plasticity reveals a functional specialization of ascending axon and parallel fiber synapses to cerebellar Purkinje cells. *J Neurosci,* 26**,** 5153-9.

SINGEWALD, N. & PHILIPPU, A. 1998. Release of neurotransmitters in the locus coeruleus. *Prog Neurobiol,* 56**,** 237-67.

SMITH, A. M. 1970. The effects of rubral lesions and stimulation on conditioned forelimb flexion responses in the cat. *Physiol Behav,* 5**,** 1121-1126.

STEINDLER, D. A. 1981. Locus coeruleus neurons have axons that branch to the forebrain and cerebellum. *Brain Res,* 223**,** 367-73.

STRADER, C. D., PICKEL, V. M., JOH, T. H., STROHSACKER, M. W., SHORR, R. G., LEFKOWITZ, R. J. & CARON, M. G. 1983. Antibodies to the beta-adrenergic receptor: attenuation of catecholamine-sensitive adenylate cyclase and demonstration of postsynaptic receptor localization in brain. *Proc Natl Acad Sci U S A,* 80**,** 1840-4.

STRAUBE, T. & FREY, J. U. 2003. Involvement of β-adrenergic receptors in protein synthesis-dependent late long-term potentiation (LTP) in the dentate gyrus of freely moving rats: the critical role of the LTP induction strength. *Neuroscience,* 119**,** 473-479.

SUGIHARA, I. & SHINODA, Y. 2004. Molecular, topographic, and functional organization of the cerebellar cortex: a study with combined aldolase C and olivocerebellar labeling. *J Neurosci,* 24**,** 8771-85.

SUGIHARA, I. & SHINODA, Y. 2007. Molecular, Topographic, and Functional Organization of the Cerebellar Nuclei: Analysis by Three-Dimensional Mapping of the Olivonuclear Projection and Aldolase C Labeling. *J Neurosci,* 27**,** 9696-9710.

SUGIHARA, I., WU, H. & SHINODA, Y. 1996. Morphology of axon collaterals of single climbing fibers in the deep cerebellar nuclei of the rat. *Neurosci Lett,* 217**,** 33-6.

SUGIHARA, I., WU, H. & SHINODA, Y. 1999. Morphology of single olivocerebellar axons labeled with biotinylated dextran amine in the rat. *J Comp Neurol,* 414**,** 131-48.

SUGIHARA, I., WU, H. S. & SHINODA, Y. 2001. The entire trajectories of single olivocerebellar axons in the cerebellar cortex and their contribution to Cerebellar compartmentalization. *J Neurosci,* 21**,** 7715-23.

SULLIVAN, R. M., WILSON, D. A. & LEON, M. 1989. Norepinephrine and Learning-Induced Plasticity in Infant Rat Olfactory System. *J Neurosci,* 9**,** 3998-4006.

SUR, C., BETZ, H. & SCHLOSS, P. 1996. Immunocytochemical detection of the serotonin transporter in rat brain. *Neuroscience,* 73**,** 217-231.

SUTIN, J. & MINNEMAN, K. P. 1985. Adrenergic beta receptors are not uniformly distributed in the cerebellar cortex. *J Comp Neurol,* 236**,** 547-54.

SVENSSON, P., BENGTSSON, F. & HESSLOW, G. 2006. Cerebellar inhibition of inferior olivary transmission in the decerebrate ferret. *Exp Brain Res,* 168**,** 241-53.

SWANSON, L. W. & HARTMAN, B. K. 1975. The central adrenergic system. An immunofluorescence study of the location of cell bodies and their efferent connections in the rat utilizing dopamine-beta-hydroxylase as a marker. *J Comp Neurol,* 163**,** 467-505.

SYKOVÁ, E. & NICHOLSON, C. 2008. Diffusion in Brain Extracellular Space. *Physiological reviews,* 88**,** 1277-1340.

TABER, K. H. & HURLEY, R. A. 2014. Volume Transmission in the Brain: Beyond the Synapse. *The Journal of Neuropsychiatry and Clinical Neurosciences,* 26**,** iv-4.

TAKEUCHI, Y., KIMURA, H. & SANO, Y. 1982. Immunohistochemical demonstration of serotonin-containing nerve fibers in the cerebellum. *Cell Tissue Res,* 226**,** 1-12.

TALLEY, E. M., ROSIN, D. L., LEE, A., GUYENET, P. G. & LYNCH, K. R. 1996. Distribution of alpha 2A-adrenergic receptor-like immunoreactivity in the rat central nervous system. *J Comp Neurol,* 372**,** 111-34.

TAVARES, A., HANDY, D. E., BOGDANOVA, N. N., ROSENE, D. L. & GAVRAS, H. 1996. Localization of α_{2A}- and α_{2B}-Adrenergic Receptor Subtypes in Brain. *Hypertension,* 27**,** 449-455.

TELLEZ, R., GOMEZ-VIQUEZ, L. & MENESES, A. 2012. GABA, glutamate, dopamine and serotonin transporters expression on memory formation and amnesia. *Neurobiol Learn Mem,* 97**,** 189-201.

TEN BRINKE, M. M., BOELE, H. J., SPANKE, J. K., POTTERS, J. W., KORNYSHEVA, K., WULFF, P., AC, I. J., KOEKKOEK, S. K. & DE ZEEUW, C. I. 2015. Evolving Models of Pavlovian Conditioning: Cerebellar Cortical Dynamics in Awake Behaving Mice. *Cell Rep,* 13**,** 1977-88.

TEUNE, T. M., VAN DER BURG, J., DE ZEEUW, C. I., VOOGD, J. & RUIGROK, T. J. H. 1998. Single Purkinje cell can innervate multiple classes of projection neurons in the cerebellar nuclei of the rat: A light microscopic and ultrastructural triple-tracer study in the rat. *J Comp Neurol,* 392**,** 164-178.

TEUNE, T. M., VAN DER BURG, J., DE ZEEUW, C. I., VOOGD, J. & RUIGROK, T. J. H. 1998. Single Purkinje cell can innervate multiple classes of projection neurons in the cerebellar nuclei of the rat: A light microscopic and ultrastructural triple-tracer study in the rat. *J Comp Neurol,* 392**,** 164-178.

THACH, W. T. 1967. Somatosensory receptive fields of single units in cat cerebellar cortex. *J Neurophysiol,* 30**,** 675-696.

THACH, W. T. 1998a. A role for the cerebellum in learning movement coordination. *Neurobiol Learn Mem,* 70**,** 177-88.

THACH, W. T. 1998b. What is the role of the cerebellum in motor learning and cognition? *Trends Cogn Sci,* 2**,** 331-7.

THELLUNG, S., BARZIZZA, A., MAURA, G. & RAITERI, M. 1993. Serotonergic inhibition of the mossy fibre--granule cell glutamate transmission in rat cerebellar slices. *Naunyn Schmiedebergs Arch Pharmacol,* 348**,** 347-51.

THEODOSIS, D. T. & POULAIN, D. A. 1993. Activity-dependent neuronal-glial and synaptic plasticity in the adult mammalian hypothalamus. *Neuroscience,* 57**,** 501-535.

THOMPSON, R. F. 1976. The search for the engram. *Am Psychol,* 31**,** 209-27.

TOYAMA, K., TSUKAHABA, N., KOSAKA, K. & MATSUNAMI, K. Synaptic excitation of red nucleus neurones by fibres from interpositus nucleus. *Exp Brain Res,* 11**,** 187-198.

TROTT, J. R. & ARMSTRONG, D. M. 1987a. The cerebellar corticonuclear projection from lobule Vb/c of the cat anterior lobe: a combined electrophysiological and autoradiographic study. I. Projections from the intermediate region. *Exp Brain Res,* 66**,** 318-38.

TROTT, J. R. & ARMSTRONG, D. M. 1987b. The cerebellar corticonuclear projection from lobule Vb/c of the cat anterior lobe: a combined electrophysiological and autoradiographic study. II. Projections from the vermis. *Exp Brain Res,* 68**,** 339-54.

TROUILLAS, P., BRUDON, F. & ADELEINE, P. 1988. Improvement of cerebellar ataxia with levorotatory form of 5-hydroxytryptophan: A double-blind study with quantified data processing. *Archives of Neurology,* 45**,** 1217-1222.

TULLY, K. & BOLSHAKOV, V. Y. 2010. Emotional enhancement of memory: how norepinephrine enables synaptic plasticity. *Mol Brain,* 3**,** 15.

UCHIDA, S., UMEEDA, H., KITAMOTO, A., MASUSHIGE, S. & KIDA, S. 2007. Chronic reduction in dietary tryptophan leads to a selective impairment of contextual fear memory in mice. *Brain Res,* 1149**,** 149-156.

UNGERSTEDT, U. 1971. Stereotaxic mapping of the monoamine pathways in the rat brain. *Acta Physiol Scand Suppl,* 367**,** 1-48.

UUSISAARI, M. & DE SCHUTTER, E. 2011. The mysterious microcircuitry of the cerebellar nuclei. *J Physiol,* 589**,** 3441-57.

UUSISAARI, M., OBATA, K. & KNÖPFEL, T. 2007. Morphological and Electrophysiological Properties of GABAergic and Non-GABAergic Cells in the Deep Cerebellar Nuclei. *J Neurophysiol,* 97**,** 901-911.

VAN DER WANT, J. J. L., WIKLUND, L., GUEGAN, M., RUIGROK, T. & VOOGD, J. 1989. Anterograde tracing of the rat olivocerebellar system with phaseolus vulgaris leucoagglutinin (PHA-L). Demonstration of climbing fiber collateral innervation of the cerebellar nuclei. *J Comp Neurol,* 288**,** 1-18.

VAN HAM, J. J. & YEO, C. H. 1992. Somatosensory Trigeminal Projections to the Inferior Olive, Cerebellum and other Precerebellar Nuclei in Rabbits. *Eur J Neurosci,* 4**,** 302-317.

VAN HAM, J. J. & YEO, C. H. 1996. Trigeminal inputs to eyeblink motoneurons in the rabbit. *Exp Neurol,* 142**,** 244-57.

VANKOV, A., HERVÉ-MINVIELLE, A. & SARA, S. J. 1995. Response to Novelty and its Rapid Habituation in Locus Coeruleus Neurons of the Freely Exploring Rat. *Eur J Neurosci,* 7**,** 1180-1187.

VARGOVÁ, L. & SYKOVÁ, E. 2014. Astrocytes and extracellular matrix in extrasynaptic volume transmission. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 369**,** 20130608.

VEASEY, S. C., FORNAL, C. A., METZLER, C. W. & JACOBS, B. L. 1995. Response of serotonergic caudal raphe neurons in relation to specific motor activities in freely moving cats. *J Neurosci,* 15**,** 5346-59.

VERKHRATSKY, A., ORKAND, R. K. & KETTENMANN, H. 1998. Glial Calcium: Homeostasis and Signaling Function. *Physiological Reviews,* 78**,** 99-141.

VERNEY, C., GRZANNA, R. & FARKAS, E. 1982. Distribution of dopamine-beta-hydroxylase-like immunoreactive fibers in the rat cerebellar cortex during ontogeny. *Dev Neurosci,* 5**,** 369-74.

VIANNA, M. R., SZAPIRO, G., MCGAUGH, J. L., MEDINA, J. H. & IZQUIERDO, I. 2001. Retrieval of memory for fear-motivated training initiates extinction requiring protein synthesis in the rat hippocampus. *Proc Natl Acad Sci U S A,* 98**,** 12251-4.

VITALE, F., MATTEI, C., CAPOZZO, A., PIETRANTONI, I., MAZZONE, P. & SCARNATI, E. 2016. Cholinergic excitation from the pedunculopontine tegmental nucleus to the dentate nucleus in the rat. *Neuroscience,* 317**,** 12-22.

VIZUETE, M. L., TRAIFFORT, E., BOUTHENET, M. L., RUAT, M., SOUIL, E., TARDIVEL-LACOMBE, J. & SCHWARTZ, J. C. 1997. Detailed mapping of the histamine H2 receptor and its gene transcripts in guinea-pig brain. *Neuroscience,* 80**,** 321-343.

VOOGD, J. 1969. The importance of fibre connections in the comparative anatomy of the mammalian cerebellum. *In:* LLINAS, R. (ed.) *Neurobiology of Cerebellar Evolution and Development: proceedings of the first International symposium of the Institute for Biomedical Research.* Chicago, Ill: American Medical Association.

VOOGD, J. 2011. Cerebellar zones: a personal history. *Cerebellum,* 10**,** 334-50.

VOOGD, J. & BIGARÉ, F. 1980. Topographical distribution of olivary and cortico nuclear fibres in the cerebellum: a review. *In:* COURVILLE, J., DE MONTIGNY, C. & LAMARRE, Y. (eds.) *The Inferior Olivary Nucleus Anatomy and Physiology.* New York: Raven.

VOOGD, J. & GLICKSTEIN, M. 1998. The anatomy of the cerebellum. *Trends Neurosci,* 21**,** 370-5.

VOOGD, J. & RUIGROK, T. J. 2004. The organization of the corticonuclear and olivocerebellar climbing fiber projections to the rat cerebellar vermis: the congruence of projection zones and the zebrin pattern. *J Neurocytol,* 33**,** 5-21.

VOOGD, J., SHINODA, Y., RUIGROK, T. J. H. & SUGIHARA, I. 2013. Cerebellar Nuclei and the Inferior Olivary Nuclei: Organization and Connections. *In:* MANTO, M., SCHMAHMANN, J. D., ROSSI, F., GRUOL, D. L. & KOIBUCHI, N.

(eds.) *Handbook of the Cerebellum and Cerebellar Disorders.* Dordrecht: Springer Netherlands.

WALKER, J. J., BISHOP, G. A., HO, R. H. & KING, J. S. 1988. Brainstem origin of serotonin- and enkephalin-immunoreactive afferents to the opossum's cerebellum. *J Comp Neurol,* 276**,** 481-497.

WALLING, S. G. & HARLEY, C. W. 2004. Locus Ceruleus Activation Initiates Delayed Synaptic Potentiation of Perforant Path Input to the Dentate Gyrus in Awake Rats: A Novel β-Adrenergic- and Protein Synthesis-Dependent Mammalian Plasticity Mechanism. *J Neurosci,* 24**,** 598-604.

WANAKA, A., KIYAMA, H., MURAKAMI, T., MATSUMOTO, M., KAMADA, T., MALBON, C. C. & TOHYAMA, M. 1989. Immunocytochemical localization of beta-adrenergic receptors in the rat brain. *Brain Res,* 485**,** 125-40.

WANG, F., XU, Q., WANG, W., TAKANO, T. & NEDERGAARD, M. 2012. Bergmann glia modulate cerebellar Purkinje cell bistability via Ca2+-dependent K+ uptake. *Proc Natl Acad Sci U S A,* 109**,** 7911-7916.

WANG, G. S., CHANG, N. C., WU, S. C. & CHANG, A. C. 2002. Regulated expression of alpha2B adrenoceptor during development. *Dev Dyn,* 225**,** 142-52.

WATANABE, M. 2002. Glial processes are glued to synapses via Ca(2+)-permeable glutamate receptors. *Trends Neurosci,* 25**,** 5-6.

WATSON, M. & MCELLIGOTT, J. G. 1983. 6-OHDA induced effects upon the acquisition and performance of specific locomotor tasks in rats. *Pharmacol Biochem Behav,* 18**,** 927-34.

WATSON, M. & MCELLIGOTT, J. G. 1984. Cerebellar norepinephrine depletion and impaired acquisition of specific locomotor tasks in rats. *Brain Res,* 296**,** 129-38.

WELSH, J. P. & HARVEY, J. A. 1991. Pavlovian conditioning in the rabbit during inactivation of the interpositus nucleus. *J Physiol,* 444**,** 459-80.

WELSH, J. P. & HARVEY, J. A. 1992. The role of the cerebellum in voluntary and reflexive movements: history and current status. *In:* LLINAS, R. & SOTELO, C. (eds.) *The cerebellum revisited.* New York: Springer-Verlag.

WELSH, J. P. & HARVEY, J. A. 1998. Acute inactivation of the inferior olive blocks associative learning. *Eur J Neurosci,* 10**,** 3321-3332.

WELSH, S. E., ROMANO, A. G. & HARVEY, J. A. 1998. Effects of serotonin 5-HT(2A/2C) antagonists on associative learning in the rabbit. *Psychopharmacology (Berl),* 137**,** 157-63.

WILSON, K. M. & MINNEMAN, K. P. 1989. Regional Variations in α_1-Adrenergic Receptor Subtypes in Rat Brain. *J Neurochem,* 53**,** 1782-1786.

WINSKY, L. & HARVEY, J. A. 1992. 6-Hydroxydopamine induced impairment of Pavlovian conditioning in the rabbit. *Neurochem Res.,* 17**,** 415-422.

WINTER, J. C. & PETTI, D. T. 1987. The effects of 8-hydroxy-2-(di-n-propylamino)tetralin and other serotonergic agonists on performance in a radial maze: a possible role for 5-HT_{1A} receptors in memory. *Pharmacol Biochem Behav,* 27**,** 625-8.

WOODRUFF-PAK, D. S., LAVOND, D. G. & THOMPSON, R. F. 1985. Trace conditioning abolished by cerebellar nuclear lesions but not lateral cerebellar cortex aspirations. *Brain Res,* 348**,** 249-260.

WOODS, S., CLARKE, N. N., LAYFIELD, R. & FONE, K. C. 2012. 5-HT(6) receptor agonists and antagonists enhance learning and memory in a conditioned emotion response paradigm by modulation of cholinergic and glutamatergic mechanisms. *Br J Pharmacol,* 167**,** 436-49.

WOODWARD, D. J., MOISES, H. C., WATERHOUSE, B. D., HOFFER, B. J. & FREEDMAN, R. 1979. Modulatory actions of norepinephrine in the central nervous system. *Fed Proc,* 38**,** 2109-16.

WU, H. S., SUGIHARA, I. & SHINODA, Y. 1999. Projection patterns of single mossy fibers originating from the lateral reticular nucleus in the rat cerebellar cortex and nuclei. *J Comp Neurol,* 411**,** 97-118.

YAMAMOTO, T., ISHIKAWA, M. & TANAKA, C. 1977. Catecholaminergic terminals in the developing and adult rat cerebellum. *Brain Res,* 132**,** 355-61.

YEH, H. H. & WOODWARD, D. J. 1983. Beta-1 adrenergic receptors mediate noradrenergic facilitation of Purkinje cell responses to gamma-aminobutyric acid in cerebellum of rat. *Neuropharmacology,* 22**,** 629-39.

YEO, C. H., HARDIMAN, M. J. & GLICKSTEIN, M. 1984. Discrete lesions of the cerebellar cortex abolish the classically conditioned nictitating membrane response of the rabbit. *Behav Brain Res,* 13**,** 261-6.

YEO, C. H., HARDIMAN, M. J. & GLICKSTEIN, M. 1985a. Classical conditioning of the nictitating membrane response of the rabbit. I. Lesions of the cerebellar nuclei. *Exp Brain Res,* 60**,** 87-98.

YEO, C. H., HARDIMAN, M. J. & GLICKSTEIN, M. 1985b. Classical conditioning of the nictitating membrane response of the rabbit. II. Lesions of the cerebellar cortex. *Exp Brain Res,* 60**,** 99-113.

YEO, C. H., HARDIMAN, M. J. & GLICKSTEIN, M. 1985c. Classical conditioning of the nictitating membrane response of the rabbit. III. Connections of cerebellar lobule HVI. *Exp Brain Res,* 60**,** 114-26.

YEO, C. H. & HESSLOW, G. 1998. Cerebellum and conditioned reflexes. *Trends Cogn Sci,* 2**,** 322-30.

YEO, C. H., LOBO, D. H. & BAUM, A. 1997. Acquisition of a new-latency conditioned nictitating membrane response--major, but not complete, dependence on the ipsilateral cerebellum. *Learn Mem,* 3**,** 557-77.

YU, J. 1972. The pathway mediating ipsilateral limb hyperflexion after cerebellar paravermal cortical ablation or cooling in cats. *Exp Neurol,* 36**,** 549-562.

YUAN, Q., HARLEY, C. W., BRUCE, J. C., DARBY-KING, A. & MCLEAN, J. H. 2000. Isoproterenol Increases CREB Phosphorylation and Olfactory Nerve–Evoked Potentials in Normal and 5-HT-Depleted Olfactory Bulbs in Rat Pups Only at Doses That Produce Odor Preference Learning. *Learn Mem,* 7**,** 413-421.

YUAN, Q., HARLEY, C. W. & MCLEAN, J. H. 2003. Mitral Cell β_1 and 5-HT_{2A} Receptor Colocalization and cAMP Coregulation: A New Model of Norepinephrine-Induced Learning in the Olfactory Bulb. *Learn Mem,* 10**,** 5-15.

ZBARSKA, S., BLOEDEL, J. R. & BRACHA, V. 2008. Cerebellar Dysfunction Explains the Extinction-Like Abolition of Conditioned Eyeblinks After NBQX Injections in the Inferior Olive. *J Neurosci,* 28**,** 10-20.

ZHANG, J., MULLER, J. F. & MCDONALD, A. J. 2013. Noradrenergic innervation of pyramidal cells in the rat basolateral amygdala. *Neuroscience,* 228**,** 395-408.

ZHOU, H.-C., SUN, Y.-Y., CAI, W., HE, X.-T., YI, F., LI, B.-M. & ZHANG, X.-H. 2013. Activation of β_2-adrenoceptor enhances synaptic potentiation and behavioral memory via cAMP-PKA signaling in the medial prefrontal cortex of rats. *Learn Mem,* 20**,** 274-284.

ZILLES, K. & AMUNTS, K. 2010. Centenary of Brodmann's map - conception and fate. *Nat Rev Neurosci,* 11**,** 139-145.